American Journal of
Veterinary Research

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A Publication of the American Veterinary Medical Association

Volume XV JANUARY, 1954 No. 54

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Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953. Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors. Subscription price: $5.00 per year for United States and countries affiliated with the Pan American Postal Union; Canada and foreign countries, $5.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $15.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

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VOL. XV JANUARY, 1954 No. 54

Experimental Hepatic Necrobacillosis in Beef Cattle

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Fort Collins, Colorado

HEPATIC necrobacillosis is an infectious noncontagious disease, characterized by single or multiple foci of necrosis or by abscesses, occurring in cattle, especially beef breeds, and is caused by *Spherophorus necrophorus*.

In early stages of acute multiple abscess formation, affected animals are sick clinically and may die. In chronic stages of multiple abscess formation and throughout the entire course of single abscess formation, affected animals are not sick clinically and, consequently, continue to eat and to gain in body weight.

The disease causes economic loss to beef cattle producers and to the meat-packing industry. Losses accruing to livestock producers result from death of animals and reduction in efficiency in feed utilization. At the time of slaughter abscessed livers are condemned.

Essentially, no positive information substantiated by experimental data is available on: (1) the route by which *S. necrophorus* enters the liver; (2) stages through which abscesses pass as they develop and heal; and (3) the duration of abscesses.

OBJECTIVES

This investigation was undertaken with the hope that a thorough understanding of the pathogenesis of liver abscesses would make possible reduction of the incidence of the disease. Specific objectives of the study were the determination of:

1) The ability of intraportal inoculations of viable pure cultures of *S. necrophorus* to produce liver abscesses.
2) Morphological changes in abscesses as they develop and heal.
3) The duration of liver abscesses.
4) The ability of polyvalent culture filtrates of *S. necrophorus* to immunize ruminants against liver abscesses.

REVIEW OF LITERATURE

The Report of the United States Department of Agriculture, Bureau of Animal Industry, Meat Inspection Service [14] indicated that for the fiscal year 1952, 7.85 per cent of livers from adult cattle slaughtered were condemned for abscesses, and 0.66 per cent of livers of all calves slaughtered were condemned for abscesses.

Schuetz [13] made the first description of bovine hepatic necrobacillosis. McFadyean [9] studied 4 cases of bovine and 1 case of ovine hepatic necrobacillosis from 1886 to 1890. He assumed that the organisms penetrated the portal system from areas of inflammation along the intestines.

According to Cohrs [2] and Joest,[7] abscesses commonly occurred in cattle and uncommonly in other species of domestic animals. They stated that abscesses were caused by *S. necrophorus*, that presumably the organisms were normally present in the alimentary tract, that they entered the portal system through areas of inflammation, and that in some cases the organisms entered the liver via the umbilicus at birth, or via the hepatic artery as a result of septicemia. Abscesses occurred as single or multiple lesions. Chronic lesions contained sequestered necrotic tissue surrounded by pus and fibrous connective tissue.

Newsom [10] and Madin [8] studied the bacteriology of bovine liver abscesses and found that 84 per cent contained pure cultures of *Actinomyces necrophorus;* 13 per cent contained *A. necrophorus* plus some other contaminant; and 3 per cent did not contain viable *A. necrophorus*.

Yamamota [15] studied the pathology of hepatic necrobacillosis. Seventeen abscessed livers were collected, presumably in varying stages of development, and from them *A. necrophorus* was iso-

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A portion of this study was supported by a Ralston Purina Research Fellowship, conducted at the Institute of Experimental Medicine of the Mayo Foundation, and submitted by the senior author to the graduate school, University of Minnesota, St. Paul, in partial fulfillment of the requirements for the degree of doctor of philosophy.

lated. On the basis of contents, the abscesses were classified as (1) sequestered and (2) softened.

Frederick [3] studied reticulums of 2,042 slaughtered dairy cattle which had abscessed livers; of these, 5.9 per cent were caused by penetration of the reticulum by foreign bodies. He also studied 2,042 reticulums from fat beef cattle which had abscessed livers; of these, 9.2 per cent were caused by penetration of the reticulum by foreign bodies.

Jensen, Frey, Cross, and Connell [6] studied the time of development of liver abscesses. Cattle were placed in a commercial feedlot and fattened on a commercial ration. A random sample of animals was slaughtered at the beginning of fattening and at intervals of forty days throughout the period of fattening. The first abscesses were found in the animals which had been on feed for eighty days. Experimental data suggested that telangiectasis predisposed liver tissue to abscesses.

Robinson, Jasper, and Guilbert [11] injected viable cultures of *S. necrophorus* into the jugular vein of 4 cattle and failed to produce liver abscesses. They suggested that continuous introduction of the organism into the peripheral venous system from such lesions as those in foot rot was necessary to produce liver abscesses.

Beaveridge[*1] attempted to immunize rabbits. Six animals were given two immunizing doses of formalized anaculture. Subsequently, the rabbits reacted to a challenge dose of viable *S. necrophorus* as unimmunized animals.[*]

So far as could be determined from the literature, hepatic necrobacillosis had not been produced experimentally, and intraportal inoculation of experimental animals with viable *S. necrophorus* had not been performed.

MATERIALS AND METHODS

To obtain information on the pathogenesis of liver abscesses, two phases of the disease were studied: (1) experimental production of liver abscesses by intraportal inoculation of cattle and sheep with viable, pure cultures of *S. necrophorus*, and (2) immunization against experimental liver abscesses.

EXPERIMENTAL PRODUCTION OF LIVER ABSCESSES

In an effort to produce liver abscesses, viable fluid culture of *S. necrophorus* was inoculated intraportally into experimental animals. After inoculation, autopsies were performed at varying intervals to determine the duration of abscesses and the morphological changes through which the abscesses pass as they develop and heal.

Experimental Animals. — Cattle. — Thirty-three cattle were used as experimental animals. All were healthy and in good physical condition at the beginning of the experiment. For several months prior to being used experimentally, they had been maintained on a ration of alfalfa hay. Males,

[*] For a comprehensive review of literature, *see* Jensen.[5]

TABLE 1—Intraportal Inoculation of 33 Cattle with Viable Spherophorus Necrophorus

Animal (No.)	Breed	Age (mo.)	Sex	Inoculum (ml.)	Strains in inoc.	No. liver abscesses	Days from inocul. to autopsy
50R-8	Guernsey	7	Mc	2.0	1	17	1.5
50R-5	Aberdeen Angus	5	F	2.0	1	0	4
50R-1	Jersey	6	Mc	2.0	1	1	6
50R-4	Hol.-Fries.	7	M	2.0	1	40	7
50R-11	Shorthorn	5	Mc	2.0	1	400	7
49R-57	Shorthorn	10	Mc	0.5	1	1	8
49R-59	Guernsey	10	Mc	0.5	1	0	8
50R-6	Aberdeen Angus	5	F	2.0	1	150	8
50R-2	Jersey	8	F	2.0	1	2	10
49R-51	Hol.-Fries.	8	F	0.2	1	0	12
49R-65	Hol.-Fries.	8	Mc	0.2	1	0	12
49R-52	Shorthorn	9	F	0.3	1	0	12
49R-66	Shorthorn	6	F	0.3	1	1	12
50R-3	Guernsey	8	M	2.0	1	2	12
49R-54	Guernsey	10	Mc	0.5	1	0	16
49R-56	Hol.-Fries.	10	Mc	0.5	1	0	16
50R-9	Guernsey	12	Mc	2.0	1	4	16
50R-12	Hol.-Fries.	6	Mc	2.0	1	30	20
48R-15	Hol.-Fries.	7	....	0.1	1	0	24
48R-14	Hol.-Fries.	6	....	0.1	1	9	27
48R-10	Hol.-Fries.	5	....	0.1	1	25	29
51R-13	Hol.-Fries.	8	Mc	1.0	1	70	29
48R-13	Hol.-Fries.	6	....	0.1	1	10	35
48R-12	Hol.-Fries.	6	....	0.1	1	1	38
50R-14	Hol.-Fries.	9	M	2.0	1	4(s)	45
50R-13	Guernsey	9	M	2.0	1	2(s)	46
51R-14	Guernsey	7	Mc	1.0	1	2	91
52R-1	Hol.-Fries.	9	Mc	4.0	14	10	100
52R-2	Hol.-Fries.	9	Mc	4.0	14	0	100
52R-3	Hol.-Fries.	9	Mc	4.0	14	6(s)	151
52R-4	Guernsey	9	Mc	4.0	14	1	151
52R-5	Guernsey	9	Mc	4.0	14	0	180
52R-6	Guernsey	9	Mc	4.0	14	13(s)	183

Mc—castrated male; (s)—scars.

steers, and females were included. Ages varied from 5 to 12 months. Breeds included Holstein-Friesians, Guernseys, Jerseys, Shorthorns, Aberdeen Angus, and Herefords. Subsequent to intraportal inoculation, the animals were killed and autopsied. The interval between inoculation and autopsy varied from one and a half to 183 days.

Sheep.—Six healthy adult Hampshires were used as experimental animals. Both males and females were included. Prior to, and during the experiment, they were maintained on pasture and a small grain supplement. Their physical condition was excellent. Following intraportal inoculations, the animals were killed and autopsied. The interval between inoculation and autopsy varied from three to twenty days.

In addition, 14 sheep were inoculated intraportally to test the ability of polyvalent culture filtrates of *S. necrophorus* to immunize against experimental infection. *See* section on immunization against liver abscesses.

Swine.—Six young, healthy Duroc Jersey pigs were used as experimental animals. Both sexes were included and their physical condition was medium. Weights varied from 15 to 35 pounds. Prior to, and during the experiment, the animals were maintained on pasture and grain supplement. Subsequent to intraportal inoculation with viable culture of *S. necrophorus*, they were euthanized and autopsied. The interval between inoculation and autopsy varied from five to twenty days.

Inoculum.—The inoculum consisted of fluid whole culture of viable *S. necrophorus*. All strains used were isolated from bovine liver abscesses. Initial isolations and subcultures were made on Hall's brain broth[4] and on Rosenows' dextrose-brain broth.[12] Anaërobiosis was maintained.

After incubation of cultures for twenty-four hours, growth was indicated by turbidity of the supernatant fluid and production of acid and gas. The purity of cultures was determined by the following criteria: (1) strict anaërobic requirement, (2) gram-negative staining, and (3) predominance of nonbranching filaments which contained granules. All cultures which contained contaminating species of bacteria were discarded. Several subcultures without evidence of contamination were required before purity was assumed.

Only strains of recent isolation were used for inoculum. Inoculums used for early intraportal injections were composed of a single, pure strain of *S. necrophorus;* while inoculums used during the latter part of the experimental period were composed of multiple strains of *S. necrophorus.*

Inoculums were not standardized for the number of bacteria because of technical difficulties: (1) the organism is strictly anaërobic; (2) it grows poorly on solid mediums; (3) it is killed by contact with free oxygen; (4) filaments tend to segment to form multiple separate bacteria; and (5) in brain broth medium, fragments of brain tissue contribute to turbidity of the supernatant fluid. A degree of standardization was obtained by using cultures of recent isolation and of specific age.

Intraportal Inoculation.—The operation for intraportal inoculation of experimental animals required general anaesthesia with nembutal, laparotomy, and exposure of the portal vein. Standard aseptic technique was employed.

Autopsy of Experimental Animals.—After intraportal inoculation the animals were killed and autopsied at varying intervals. The liver and point of inoculation were examined for abscesses. In addition, lungs, heart, kidneys, and muscles were examined. All lesions were counted and measured. Cultures were made of representative abscesses. Remaining parts were fixed in 10 per cent formalin and sectioned.

Immunization Against Liver Abscesses

An attempt was made to evaluate the ability of culture filtrates and anacultures to immunize against liver abscesses.

TABLE 2—Intraportal Inoculation of 20 Sheep with Viable Spherophorus Necrophorus

Animal (No.)	Breed	Age (mo.)	Sex	Inoculum (ml.)	Strains in inoc.	No. liver abscesses	Days from inocul. to autopsy
1	Hampshire	2	M	1.0	4	15	3
2	Hampshire	2	M	1.0	4	20	5
4	Hampshire	3	M	4.0	4	100	5
6	Hampshire	1	F	2.0	4	30	10
122	Hampshire	3	F	2.0	4	20	15
51R-5	Cross	2	F	1.0	10	3	16
51R-6	Cross	2	F	1.0	10	6	16
51R-8	Cross	3	F	1.0	10	3	16
51R-11	Cross	2	F	1.0	10	2	16
51R-12	Cross	4	F	1.0	10	2	16
51R-15	Cross	1	Mc	1.0	10	45	16
51R-16	Cross	1	Mc	1.0	10	52	16
51R-1	Cheviot	4	F	1.0	11	2	17
51R-2	Cross	4	F	1.0	11	8	17
51R-3	Cross	4	F	1.0	11	1	17
51R-4	Cross	5	F	1.0	11	5	17
51R-7	Hampshire	3	F	1.0	11	0	17
51R-9	Hampshire	2	F	1.0	11	14	17
51R-10	Cross	4	F	1.0	11	0	17
5	Hampshire	1	F	2.0	4	8	20

Experimental Animals.—Fourteen healthy adult sheep were used. The animals were in good physical condition. They were crossbreeds and varied in age from 9 months to 5 years. Castrated males and females were included. Prior to, and during the experiment, the animals were maintained on a ration of grass pasture and alfalfa hay. The animals of each lot were approximately equal in age, sex, and breed. Lot 1, which contained 6 sheep, was given a series of intraperitoneal inoculations of an experimental vaccine. Lot 2 which contained 8 sheep was given no vaccine.

TABLE 3—Abscesses in Livers and Lungs of Sheep in Lots 1 and 2

Animal (No.)	Lot (No.)	Abscesses in liver	Abscesses in lungs
51R-1	1	2	0
51R-2	1	8	0
51R-3	1	1	0
51R-5	1	3	8
51R-7	1	0	0
51R-8	1	3	0
51R-4	2	5	0
51R-6	2	6	0
51R-9	2	14	0
51R-10	2	0	0
51R-11	2	2	0
51R-12	2	1	0
51R-15	2	45	17
51R-16	2	52	1

Vaccine.—The vaccine was composed of sterile polyvalent culture filtrates and polyvalent killed whole culture. Multiple strains of *S. necrophorus* were isolated from bovine liver abscesses. They were grown on Rosenow's dextrose-brain broth and on liver-meat broth. For each inoculation of the series, new strains of bacteria were obtained. After incubation for variable periods, the strains of whole culture were mixed together and centrifuged. The fluid part of the culture was passed through a sterile Seitz filter and tested for sterility by culturing. The filtrate was injected intraperitoneally. Each animal of lot 1 was given six injections of 10 ml. of filtrate each over a period of fifty-two days. The anaculture was prepared by formalizing whole fluid culture. After the anaculture was shown to be sterile by culturing, a single intraperitoneal injection was made.

Challenge Dose.—Twenty-seven days after the last immunizing dose, each animal of lot 1 and lot 2 was inoculated intraportally, as described above, with 1 ml. of viable fluid culture of *S. necrophorus* which was 48 hours of age and was composed of 11 strains of bovine origin. The culture medium was Rosenow's brain broth. Following inoculation the animals were returned to their original pen and ration.

Autopsy of Experimental Animals.—Sixteen days after the intraportal inoculation, all animals of lot 1 and lot 2 were killed and autopsied. The site of the intraportal inoculation, liver, lungs, heart, and kidneys, were examined for abscesses. Representative abscesses from each diseased organ were cultured for bacteria by aërobic and anaërobic methods. Remaining lesions were fixed in 10 per cent formalin. Sections were prepared and stained with hematoxylin and eosin and by Nocht's method.

Statistical Analysis.—Quantitative data obtained from examination of livers from animals of lot 1 and lot 2 were analyzed for significant differences by analysis of variance.

Results

Liver abscesses were produced experimentally in cattle and sheep by intraportal inoculation of viable *S. necrophorus*. Sheep were not protected by multiple intraperitoneal injections of sterile polyvalent culture filtrates of *S. necrophorus* against a challenge dose of viable *S. necrophorus* inoculated intraportally.

Experimental Liver Abscesses

Liver abscesses, typical of spontaneous liver abscesses from which *S. necrophorus* may be isolated, resulted from intraportal inoculation of viable *S. necrophorus* in cattle and sheep. Intraportal inoculation of swine failed to produce liver abscesses.

Cattle and Sheep.—Tables 1 and 2 present data pertaining to intraportal inoculation of cattle and sheep, respectively.

Thirty-three cattle were inoculated intraportally, and at autopsy, 69.7 per cent contained liver abscesses. The lesions varied in duration from one and a half to 183 days after inoculation. Abscess scars were

Fig. 1—Experimental hepatic necrobacillosis in bovine liver containing two foci of infection of thirty-six hours' duration. Affected tissue is necrotic.

observed in lesions which varied in duration from forty-six to 183 days. Twenty sheep were inoculated intraportally and 90 per cent contained liver lesions at autopsy. One animal died from severe and extensive hepatic nebrobacillosis.

Gross Pathology.—The size of hepatic lesions varied from 2 mm. in diameter at one and one-half days' duration to 9 cm. in diameter at 151 days' duration. All abscesses were firm and visible if located near the surface and palpable, if relatively large, when located deeply within the liver. When located near the surface, fibrinous exudate, or adhesions, were on the capsule directly over the abscess. Very young lesions were firm, pale brown in color, spherical with irregular outline, and without a capsule of fibrous connective tissue.

The cut surface varied with the duration of the abscesses. Young lesions consisted of a mass of necrotic liver tissue which was pale in color and firm to palpation. Abscesses, of eight to one hundred days' duration, contained three distinct zones from the center to the periphery: necrotic liver tissue, pus, and capsule of fibrous connective tissue. The zone of necrotic tissue reached maximum size in approximately thirty days, then diminished gradually and was not found in abscesses over one hundred days' duration. The zone of pus was readily discernible between the necrotic tissue and the capsule of fibrous connective tissue. It was narrow in young lesions but widened with age. In abscesses over one hundred days' duration, pus constituted the entire content of the abscess. The pus was tenacious, pale green, and without odor. The capsule of fibrous connective tissue was tough, pale, and glistening. It thickened continuously with age and formed the scar of healed abscesses. Figures 1 through 8 show experimental liver abscesses.

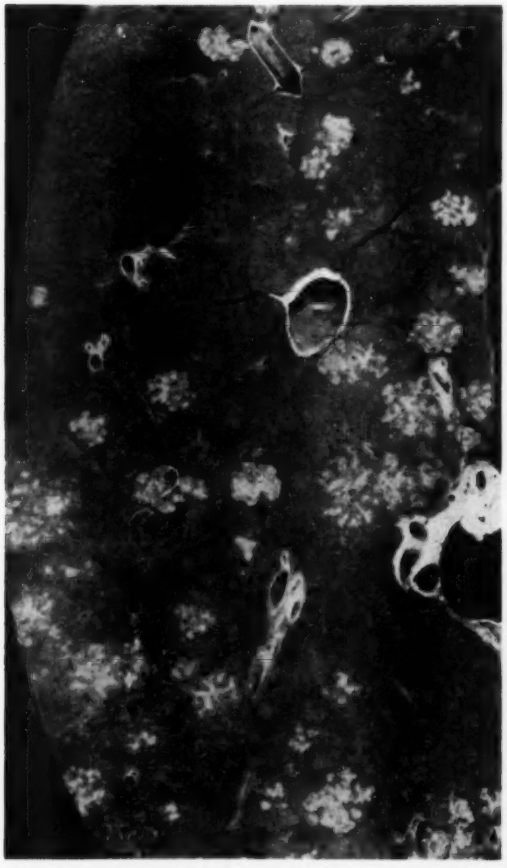

Fig. 2—Experimental hepatic necrobacillosis of sheep liver showing multiple foci of infection, of five days' duration. Foci were pale in color, firm, and irregular in outline.

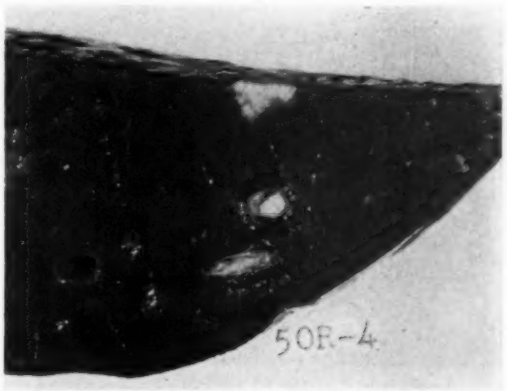

Fig. 3—Experimental hepatic necrobacillosis in bovine liver containing one focus of infection of seven days' duration.

Histopathology.—Lesions less than six days' duration showed necrosis, infiltration of leukocytes, and colonies of *S. necrophorus*. In infected areas, all parenchymal and stromal cells were dead. Structural outlines of hepatic cords, bile ducts, and vessels were discernible readily. Pyknosis, chromatolysis of nuclei, and increased affinity of cytoplasm for acid stains were generalized. Cells at the periphery of the lesion showed similar but less advanced changes. Leukocytes, especially mononu-

clear macrophages, had accumulated in the sinusoids. The majority of leukocytes was also necrotic. At the periphery of the lesion, few polymorphonuclear neutrophils were present.

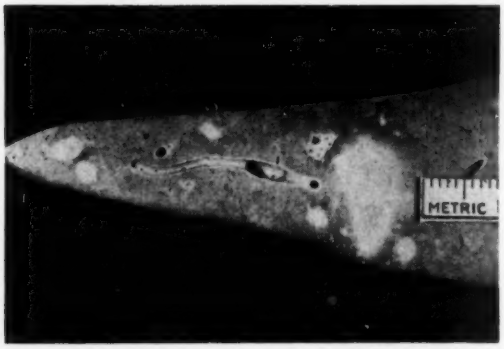

Fig. 4—Experimental hepatic necrobacillosis in bovine liver containing foci of infection of eight days duration.

Colonies of filamentous *S. necrophorus* were distributed throughout the dead tissue. Some filaments were parallel within a group; the majority of organisms was randomized in arrangement. At the periphery of the lesions, filaments of organisms were invading living tissue.

Abscesses of eight to one hundred days' duration were zoned from center to periphery: coagulative necrosis, pus, and capsule of fibrous connective tissue. Tissue in the center was necrotic liver. Hepatic cords, portal trinities, and lobules were identifiable. Much cellular detail was lost. Nuclei showed chromatolysis, and cytoplasm manifested increased affinity for acid stain. Colonies of bacteria were abundant and filamentous in young lesions. In old abscesses in the central part of the necrotic tissue, bacteria were scarce and filaments were short.

The zone of pus contained polymorphonuclear neutrophils, mononuclear macrophages, fragments of necrotic tissue, and colonies of bacteria. Except at the peripheral part of the zone, leukocytes were degenerated and necrotic. Necrotic tissue originated from the capsule of connective tissue and was added to the zone of pus. Colonies of bacteria were abundant even in old lesions. The length of filaments diminished with the age of the abscess; in old abscesses rod-shaped organisms predominated.

The capsule of fibrous connective tissue was beginning to form around one bovine abscess at six days' duration. Ovine hepatic lesions of five days' duration contained no capsule. In lesions older than six days the capsule thickened and presented gradations characteristic of granulation

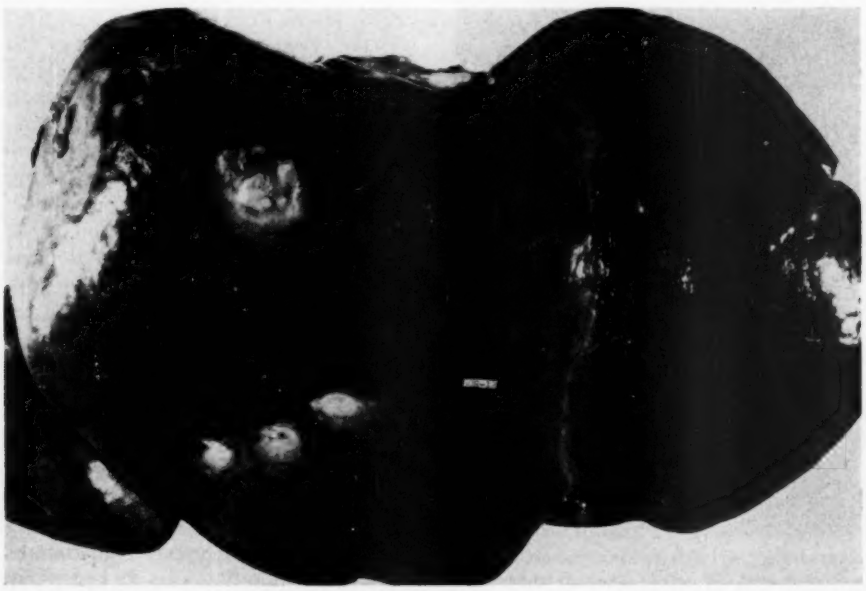

Fig. 5—Experimental hepatic necrobacillosis in bovine liver containing foci of infection of eight days' duration. The focus of infection is being converted to an abscess.

tissue. Inner and outer layers were discernible and continuous. The inner layer was composed of immature fibrocytes, leukocytes, and capillaries. The fibrocytes were stellate and contained vesicular nuclei. Collagenous fibers were scarce. Lymphocytes, mononuclear macrophages, and plasma cells were abundant. Few polymorphonuclear neutrophils were present. Lesions, several weeks of age and older, contained an abundance of fat-filled mononuclear macrophages adjacent to the zone of pus. Capillaries were numerous, and formed loops which extended toward the inner surface. The outer layer was composed of fibrous connective tissue and differed from the inner layer by gradual transition. Fibrocytes were mature, their maturity being indicated by small, compact nuclei and many collagenous fibers.

Fig. 7—Experimental liver abscess of one hundred days' duration in intact bovine liver.

Fig. 6 — Experimental hepatic abscesses of twenty-nine days' duration. In cross section of bovine liver, from center to periphery, each abscess contains necrotic liver, zone of pus, and capsule of fibrous connective tissue.

Fig. 8—Scar of experimental bovine liver abscess of 183 days' duration.

Leukocytes and capillaries were scarce. Some lymphocytes were present. At the periphery, the capsule was in direct contact with hepatic tissue. Small bile ducts had increased in number and had penetrated the peripheral part of the capsule.

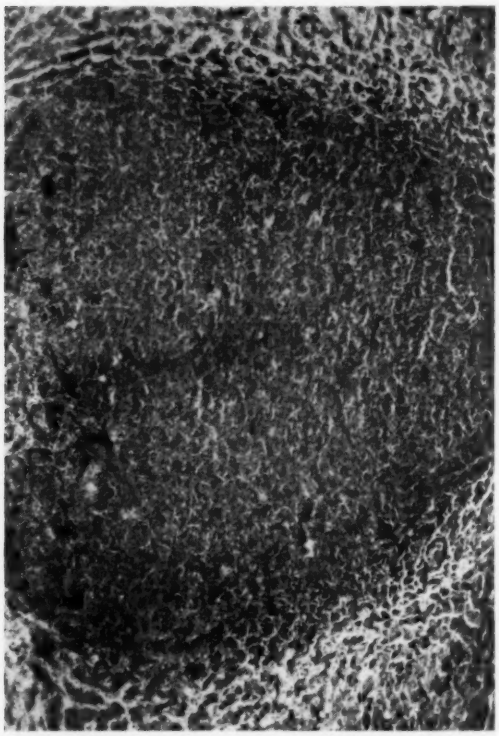

Fig. 9 — Photomicrograph. Experimental hepatic necrobacillosis of bovine liver containing focus of infection of thirty-six hours' duration. Affected tissue is necrotic. Hematoxylin and eosin stain. x 75.

Many abscesses contained secondary foci of infection with mieroabscess formation within the capsule of the primary abscess. The small secondary abscess contained pus and a peripheral wall, similar to that of primary abscesses. Figures 9, 10, 11, and 12 show histopathology of abscesses of different ages.

Immunization of Sheep Against Experimental Abscesses of the Liver

A series of intraportal injections of polyvalent culture filtrates of *S. necrophorus* failed to protect sheep against a challange dose of viable *S. necrophorus*, given intraportally. Table 3 presents the number of liver and lung abscesses, found at autopsy, of sheep given the immunizing agent and of sheep not given the immunizing agent. Analysis of variance failed to show a significant difference in susceptibility between lots 1 and 2. Bacterial cultures collected at autopsy showed the presence of pure *S. necrophorus* in most lesions.

Discussion

Of the 33 cattle which were inoculated intraportally with viable *S. necrophorus*, 69.7 per cent contained liver abscesses at the time of autopsy. Some of these abscesses could possibly have been present at the time of inoculation but this is improbable since: (1) the incidence of liver abscesses in young, unfattened cattle is low; (2) no abscesses could be palpated in the liver at the time of laparotomy for intraportal inoculation; and (3) the condition of abscesses at autopsy was consistent with

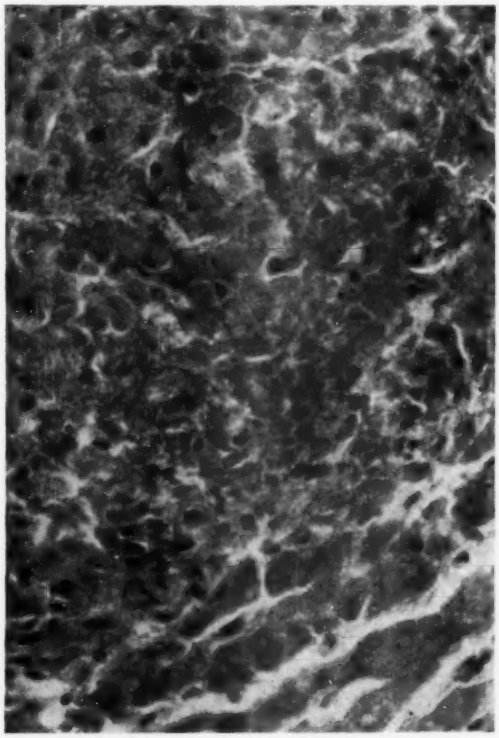

Fig. 10 — Photomicrograph. Experimental hepatic necrobacillosis of thirty-six hours' duration. Liver contains a focus of infection. Affected tissue is infiltrated with Spherophorus necrophorus. Hepatic tissue and leukocytes are necrotic. Hematoxylin and eosin stain. x 400.

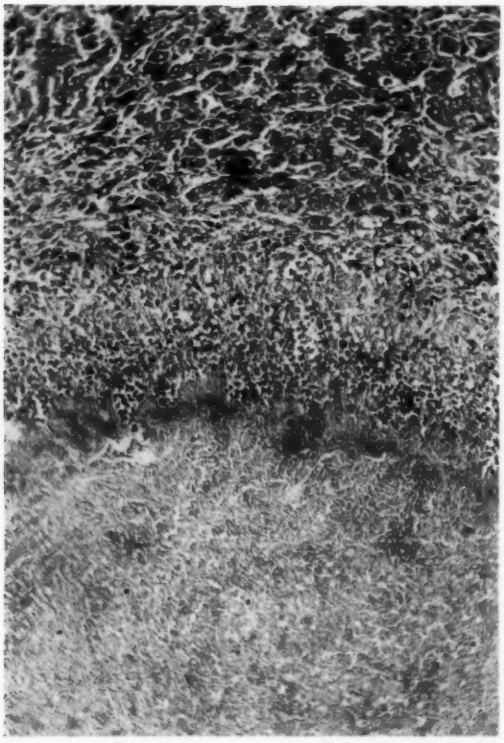

Fig. 11 — Photomicrograph. Experimental bovine liver abscess of seven days' duration. From top to bottom: normal liver, zone of inflammation, necrotic liver. Nocht's stain. x 200.

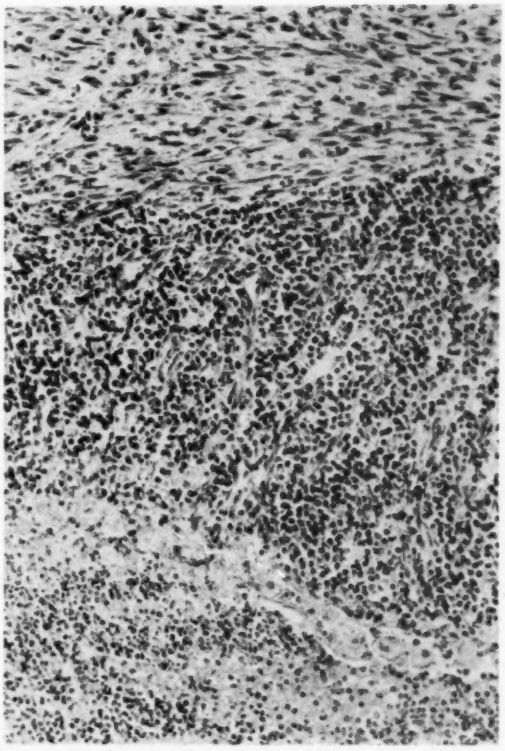

Fig. 12 — Photomicrograph. Experimental bovine liver abscess of twenty-nine days' duration. From top to bottom: fibrous connective tissue, granulation tissue, mononuclear macrophages, and pus. Nocht's stain. x 100.

expected tissue changes in lesions of an age equal to the interval from intraportal inoculation to autopsy.

When fat cattle are slaughtered, all stages in the development of liver abscesses are observed. Livers are condemned when they contain active abscesses but not because of abscess scars. Earlier experiments by Jensen, Frey, Cross, and Connell [7] indicated that spontaneous abscesses have a duration of forty to 115 days. The youngest abscess scars in this experiment were observed forty-five days after inoculation. These data indicate that most abscesses which cause condemnation of liver at slaughter originated during the feedlot period. Most abscesses which originated before the animals entered the feedlot would be in scar stages and, therefore, were not the basis for condemnation at the end of the fattening period.

Immunization was unsuccessful, using polyvalent culture filtrates as vaccine and sheep as experimental animals. In an earlier experiment, Jensen, Frey, Cross, and Connell [7] observed a new crop of abscesses develop in the feedlot in animals which contained liver abscesses when they entered the feedlot. The first attack did not immunize against the second attack. If the disease itself does not immunize the animal, there is little reason to expect that a vaccine could give protection.

SUMMARY

1) Intraportal inoculation of viable *Spherophorus necrophorus* was performed on 33 cattle, 20 sheep, and 6 swine. At autopsy, from one and a half to 183 days later, 69.7 per cent of the cattle and 90 per cent of the sheep contained liver abscesses. No abscesses were produced in swine.

2) The abscess capsule and pus began to form approximately eight days after inoculation and attained maximum size at approximately thirty days' duration. The

central necrotic liver tissue persisted for as long as one hundred days.

3) The duration of abscesses varied, scar stages being observed in lesions 45, 46, 151, and 183 days of age.

4) A series of intraperitoneal inoculations of polyvalent culture filtrates of *S. necrophorus* failed to protect sheep against liver abscesses when challenged with intraportal inoculation with viable *S. necrophorus*.

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AVMA Research Fellowships Available

The Research Council of the American Veterinary Medical Association announces the availability of a number of fellowships for postgraduate training for the academic year 1954-1955.

The recipient must be a veterinarian and a citizen of the United States or Canada. Veterinary students who expect to graduate at the end of the current school year may apply. The latest date for filing the completed application form is April 1, 1954.

Awards will be announced by May 15. The stipend will be determined in each case by the needs of the individual, the location of the school where he proposes to work, and other factors. In general, the stipends range from $100 monthly and upward.

Application blanks and other information may be obtained by writing to Dr. Robert Getty, secretary, AVMA Research Council, Department of Anatomy, Iowa State College, Ames, Iowa.

Effects of Abnormal Machine Milking on the Histopathology of Udders of First-Calf Heifers Free of Mastitis

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Storrs, Connecticut

THE ADVENT of the milking machine was accompanied by the question of its role in the bovine mastitis problem. Traumatic possibilities owing to improper use of the milking machine have long been recognized. However, to what extent abnormal machine milking *per se* contributes to the mastitis problem is not known. The present report is concerned with histological findings on the teats and udders of 11 first-calf heifers, free of mastitis, that were subjected to machine milking at different vacuum levels and milking durations.

REVIEW OF LITERATURE

Trauma of the teats,[5, 15] congested teats,[9] chapped and everted teats,[20] and clinical mastitis[7] have been reported as a result of excessive vacuum or a too long period of milking. The correct vacuum to operate a milking machine has been reported as ranging from 12 to 15 in. of mercury[4, 14, 20] and the time required to milk a cow has been reported as between three and one-half minutes[3] and five minutes.[6] However, some cows require only two minutes while others require up to seven minutes to milk out.[3] Although a long milking time may be injurious to the udder, incomplete milking of slow-milking cows may also contribute to mastitis.[26] While fast-milking cows presumably receive less irritation to the udder by the machine, they often develop mastitis since highly patent teats tend to permit infection.[8, 19]

In herds where bacterial mastitis already existed, use of the milking machine was found to aggravate the condition.[1, 2, 14, 28] Trauma to the accessory glands resulting from improper machine milking has been reported as predisposing the udder to mastitis.[22, 23]

MATERIALS AND METHODS

Eleven first-calf heifers, 3 Jerseys and 8 Holstein-Friesians, were considered free of mastitis after twice weekly observations during the first postpartum month (standardizing period). The milk samples of the third and fourth weeks contained one million or less leukocytes per milliliter and only 11 of these 192 samples contained more than 300,000 per milliliter. The teats and udders were free from visible evidence of injury.

Within breeds, in order of calving, the animals were assigned at random to one of three vacuum levels. Assignments continued until 3 Holstein-Friesian and 1 Jersey replicate were filled. Halves of udders were randomly assigned to a milking duration for first and third replicates and within a vacuum; the opposite halves for the second and fourth were assigned to the same milking duration.

Vacuum levels used were 10, 13, and 17 in. of mercury and the pulsations per minute were 48. Duration of milking was designated as "normal" and "twice normal." "Normal" was defined as the length of time required to reduce the milk flow to 0.1 lb. in a fifteen-second interval. The duration was adjusted weekly throughout the experiment. Machines employed were the goat-claw, bucket type, using two per cow.

At the fifth week, animals were milked at their assigned vacuums and times. During the thirty-day standardizing period, a vacuum of 13 in. was employed. Feeding, udder preparation, and sanitary precautions were in accord with accepted methods of dairy husbandry.

During the experimental period, the fifth through the forty-fourth weeks of lactation (comparison period), daily strip-cup and visual observations for abnormal secretion were made, and milk samples were collected aseptically from each quarter of each animal twice weekly. Portions of the unincubated samples were examined for their pH values as determined by a Beckman pH meter (model G) and for their chloride content by Rosell's method.[25] The remaining portions of the samples were incubated at 37 C. for eighteen hours. Films were then made and examined by the Prescott-Breed method[24] to determine the leukocyte count, and a small loopful of each sample was cultured on blood agar to determine the presence or absence of pathogenic bacteria. Physical examinations were made of each udder at the end of the fourth postpartum week and at the forty-fourth postpartum week according to Udall's method.[27]

Upon completion of the forty-fourth postpartum week, the 11 cows were slaughtered. The udders were weighed in the milked out state, injected with 10 per cent formalin via the teat canals, and sectioned after fixation for histological study.

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The authors are indebted to the Chas. H. Hood Dairy Foundation, Boston, Mass., and to the DeLaval Separator Co., Poughkeepsie, N. Y., for financial assistance.

TABLE 1—Occurrence of Histological Mastitis in Relation to Clinical Mastitis and Tests Made on Milk

Cow and breed	Milking duration	Vacuum in inches of Hg	Leukocytes mean log X10⁻⁴ S	Leukocytes mean log X10⁻⁴ C	Chloride / 100 mg (%) S	Chloride / 100 mg (%) C	pH mean S	pH mean C	Bacteriologic agent	Teat erosion * (Udall, 1943)	Udder fibrosis ** (Udall, 1943) S	Udder fibrosis ** (Udall, 1943) C	Clinical mastitis (cases)	Histopathological examination	Actual milk production in pounds (305 days)
	N	17	0.95	0.85	141	151	6.5	6.6	.....	3	5	5	....	Normal	5,855
1 H.-F.	2N	17	0.81	0.81	144	139	6.5	6.6	.....	6	7	7	....	Normal	
	N	13	0.92	0.83	116	132	6.7	6.7	.....	0	5	5	....	Normal, proliferation of teat orifice	7,554
2 H.-F.	2N	13	0.92	0.78	99	113	6.6	6.6	.....	0	5	7	....	Normal	
	N	10	0.85	0.83	115	134	6.6	6.6	.....	7	6	7	....	Focal acute mastitis	8,687
3 H.-F.	2N	10	0.85	0.81	112	128	6.4	6.6	.....	7	7	7	....	Normal	
	N	17	0.81	0.84	97	112	6.5	6.5	.....	4	2	5	....	Normal	9,200
4 H.-F.	2N	17	0.70	0.84	105	116	6.6	6.5	.....	3	3	6	....	Normal	
	N	10	0.81	0.85	92	114	6.1	6.6	.....	0	4	5	....	Focal acute mastitis	9,197
5 H.-F.	2N	10	0.85	0.86	92	120	6.6	6.6	.....	2	7	5	....	Proliferation of teat orifice	
	N	17	0.81	1.41	85	123	6.6	6.6	.....	2	7	5	....	Focal acute mastitis	9,484
6 H.-F.	2N	17	1.00	0.94	85	109	6.6	6.6	.....	6	7	9	....	Focal acute mastitis and thelitis	
	N	10	1.09	1.68	96	131	6.1	6.6	St. Col.	10	6	5	3	Normal	9,225
7 H.-F.	2N	10	1.12	1.20	96	116	6.1	6.6	St. Col.	10	7	4	2	Normal	
	N	13	0.81	1.76	98	119	6.4	6.6	St.	11	4	7	2	Focal acute mastitis an l thelitis	12,566
8 H.-F.	2N	13	0.74	1.39	100	113	6.3	6.6	.....	8	4	5	....	Normal	
	N	10	1.10	0.78	100	102	6.6	6.6	.....	9	4	4	....	Focal acute mastitis	6,167
9 Jer.	2N	10	1.00	0.81	104	103	6.6	6.6	.....	8	4	5	....	Normal	
	N	13	1.04	0.90	74	97	6.6	6.5	.....	2	6	6	....	Focal acute mastitis	8,352
10 Jer.	2N	13	1.20	0.82	73	94	6.6	6.5	.....	3	5	3	....	Normal	
	N	17	0.81	0.82	93	111	6.3	6.5	.....	0	6	5	....	Normal	6,840
11 Jer.	2N	17	0.85	0.82	97	114	6.4	6.5	.....	0	7	5	....	Normal	

C—comparison period; S—standardizing period; N—normal milking time; 2N—twice normal milking time; St.—hemolytic staphylococci; Col.—coliform organisms.

* Teat ends—Teats were scored negative (0), slight (1), marked (2), for the following characteristics: vegetation, proliferation, redness, and patency. These figures in the table are the accumulative score for each half.

** Udder fibrosis—Udders were scored negative (0), slight (1), slight plus (2), dense (3), dense plus (4), marked (5). These figures in the table are the accumulative score for each half.

Each teat was sectioned longitudinally to include the canal and Fürstenberg's rosette,[10] which was followed by cross sections of the remaining teat tissue. This technique usually required nine blocks. The udder was divided into three horizontal levels and sections taken from each level according to a system suggested by Morrill.[18] In this study, three to six blocks were taken per level per quarter. The supramammary lymph nodes were counted, measured, weighed, and sections placed in 10 per cent formalin. Tissues were routinely stained by Harris-hematoxylin-triosin; special stains, as special Gram's pyronin, von Gieson's and Masson's trichrome, were employed in some instances.

RESULTS

Leukocyte counts, chloride and pH values have been reported in detail elsewhere [16, 17] and only brief mention is given here.

Leukocytes.—Statistical analysis of the average logarithms of the leukocyte counts (table 1) showed no difference among the three vacuum levels. The halves milked for normal duration had a greater log count than the twice normal halves ($P < 0.05$). Investigation of time trends showed no difference in linear rates of increase for vacuum levels while the normal halves increased at a greater rate than the twice normal halves ($P < 0.01$).

Chloride.—There were no significant differences among vacuum levels (table 1). The average chloride for the normal halves was significantly greater ($P < 0.01$) than for the twice normal halves and the linear rate of increase was also greater ($P < 0.05$).

Hydrogen Ion (pH).—The average pH for the three vacuum levels as well as for milking durations did not differ significantly (table 1). In agreement with the two previous criteria, the normal halves increased at a greater linear rate ($P < 0.05$) over the lactation than the twice normal halves. [16, 17]

CLINICAL MASTITIS AND THELITIS

The types of mastitis which developed in the heifers were of bacterial origin with hemolytic staphylococci and coliform organisms being the offenders. There were no significant differences for vacuum level or milking duration for the total samples free from the mastitis organism for the comparison period.

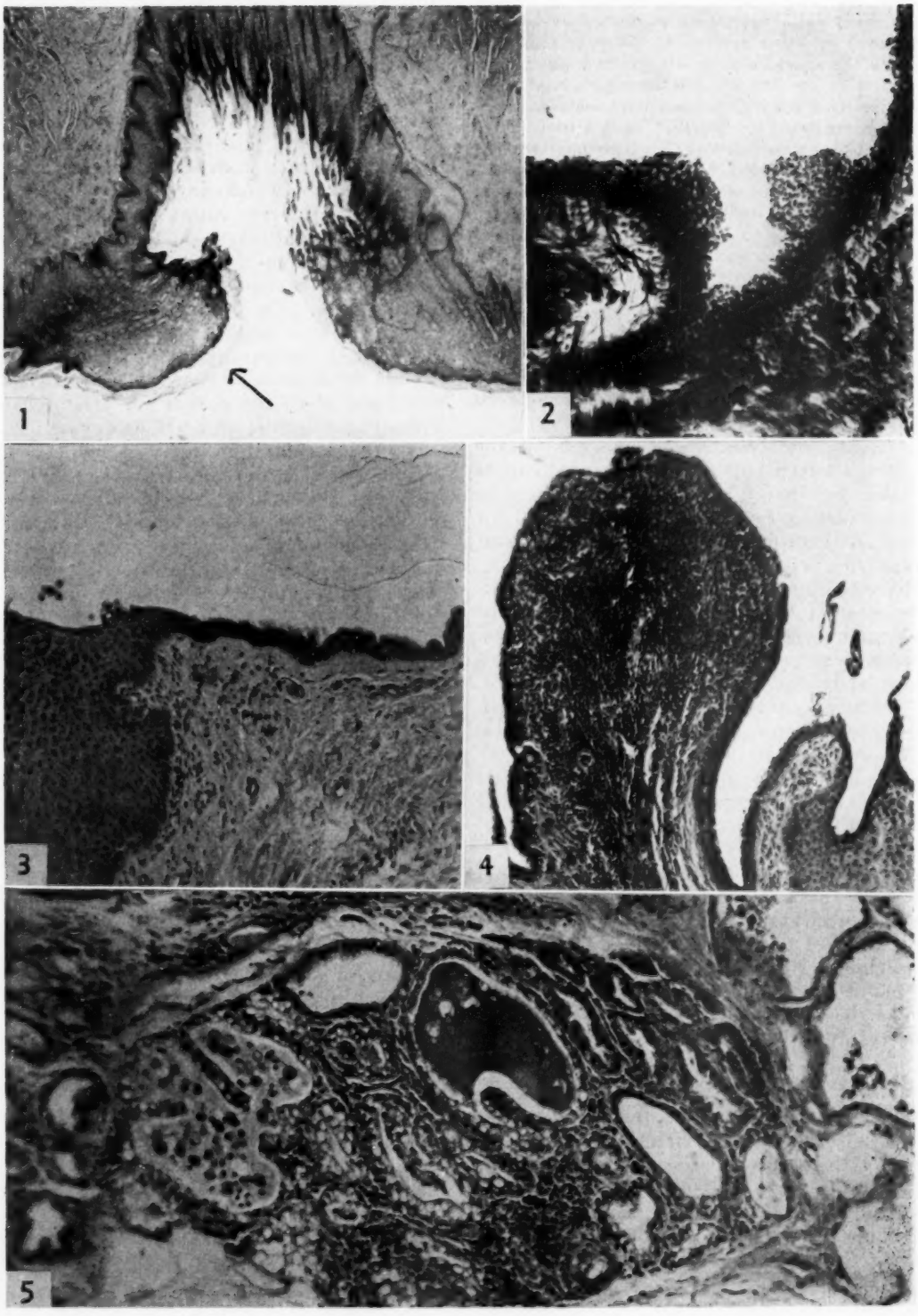
Two cows (7 and 8) showed clinical mastitis in seven instances (table 1). Although clinical mastitis occurred more frequently in normal halves, it did not explain the statistically significant higher leukocyte and chloride values of the normal halves. For example, during the thirty-day standardizing period, four of 11 normal halves had a higher mean log leukocyte count than the twice normal halves, but the number rose to ten of 11 during the comparison period. The chloride values revealed a similar trend in that seven of 11 normal halves were higher than the corresponding twice normal halves during the comparison period, while only four of 12 were higher during the standardizing period. While no satisfactory explanation could be offered, it did not seem probable that the clinical mastitis cases were the reason for the higher leukocyte, chloride, and pH values.

At the termination of the comparison period, teat ends were observed for redness, openness, vegetations, proliferations, and scored for these characteristics (table 1). Application of analysis of variance to the data showed no significant differences among the vacuum levels nor between milking durations.[16, 17]

HISTOLOGICAL EXAMINATION

Teats.—Histological examination often failed to agree with the gross findings (table 1). Teat ends which were scored normal were sometimes found histologically abnormal while the reverse was also true. For example, a teat from cow 2 was classified as normal upon visual examination, but histological examination revealed a squamous epithelial tag about the orifice (fig. 1). This hyperplastic tag was apparently of no mechanical importance, but might have been capable of holding droplets of milk which, in turn, could act as a medium for bacterial growth and thus subsequent inoculation of the udder. Histological examinations of the epithelial membrane [12] of the teats usually showed normal tissue. However, a few teats (table 1) revealed thelitis in the form of a diffuse lymphocytic infiltration of the lamina propria (fig. 3 and 4) at Fürstenberg's rosette.[10] It is probable that this was a pathological condition and suggests irritation, but correlation between the lesion and apparent misuse of the machine was not possible.

Hyperplasia or metaplasia of the teat (fig. 4) and milk cistern membrane was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not common (table 1). As in all other alterations discussed thus far, the lesions did not appear to be a result of machine milking. Actually two types of hyperplasia were recognized. Pathological hyperplasia showed increased cellular numbers of the epithelial membrane with diffuse lymphocytic infiltration of the lamina propria. Occasionally, a corpus amylaceum was trapped in the epithelial membrane (fig. 6). Benign hyperplasia (fig. 2) seemed to be a small nodule of increased epithelial cells without reaction; it was always confined to a small area. The cells were always in the same plane reducing the likelihood that the cellular group was an artifact resulting from cutting.

Udder.—Physical examination of the udders did not reveal any definite cases of mastitis, but this is understandable as all mastitic lesions observed were microscopic in size and beyond the limits of palpation (table 1). Lesions varied from foci of heterophils within the alveolar lumina to infiltration of the interalveolar tissue (fig. 5 and 8). This variation in intensity and position of the phagocyte suggested that the disease progressed from the presence of heterophils and organisms in clotted secretion (fig. 5) to the successful establishment of the organisms within the tissue. The infectious agent arrived in the alveolus by way of the ductal system, exerted positive chemotactic influence upon heterophils, and then went on to establish itself in the tissue or became overwhelmed by defensive action.

All foci seen were considered to be acute in character without visible evidence of fibrosis. The fibrosis, if it occurred, would have been rather minute and could have been overlooked by the method of taking sections. Although the lesions were the result of hemolytic staphylococci or coliform organism, the pathogenesis was similar to that described by Ibel (1904) [11] which, in essence, stated that streptococcic mastitis was a mildly acute condition which rapidly terminated in a quiescent state. It was felt that the foci in these cases terminated in a similar quiescent state owing chiefly to involution of involved alveoli. The clinical appearance of the mastitis suggested chronic mastitis, but the histological appearance was one of ever-occurring acute foci which were overcome by phagocytosis or defensive involution of the affected alveolus. Thus, organisms subsisting chiefly on the secretion of the alveolus would be deprived of the vital medium. The several histological changes were sometimes visible in a single lobulus (fig. 5).

Histologically, such glandular tissue which had involuted or overcome the organisms might be considered normal tissue. An example was cow 7 which had five attacks of clinical mastitis but at the time of slaughter the udder was without histological lesions.

The reverse of this situation, *i.e.*, mastitic lesions without evidence of abnormality in the milk, was even more frequently seen (table 1). When the minute size of the lesion is considered in relation to the udder and volume of secretion, it is not surprising

Legends for Figures on Opposite Page

All figures are photomicrographs of bovine mammary sections taken after 305 days of lactation, embedded in paraffin, and stained with hematoxylin-triosin.

Fig. 1—Sagittal section of the external end of the teat canal, from a Holstein-Friesian heifer milked at 13 in. of vacuum for normal duration. The arrow points to a tag of hyperplastic epithelium which could trap milk and bacteria. x 14.

Fig. 2—Section of the milk cistern from a Holstein-Friesian heifer milked at 10 in. of vacuum for twice normal duration. The epithelium presents a hyperplastic appearance without reaction in lamina propria. This is considered to be benign hyperplasia. x 100.

Fig. 3—Section of teat from a Holstein-Friesian heifer milked at 13 in. of vacuum for normal duration. A normal membranocutaneous junction at Fürstenberg's rosette is shown. Note that stratified squamous epithelium differentiated from the double-layered membranous epithelium. x 100.

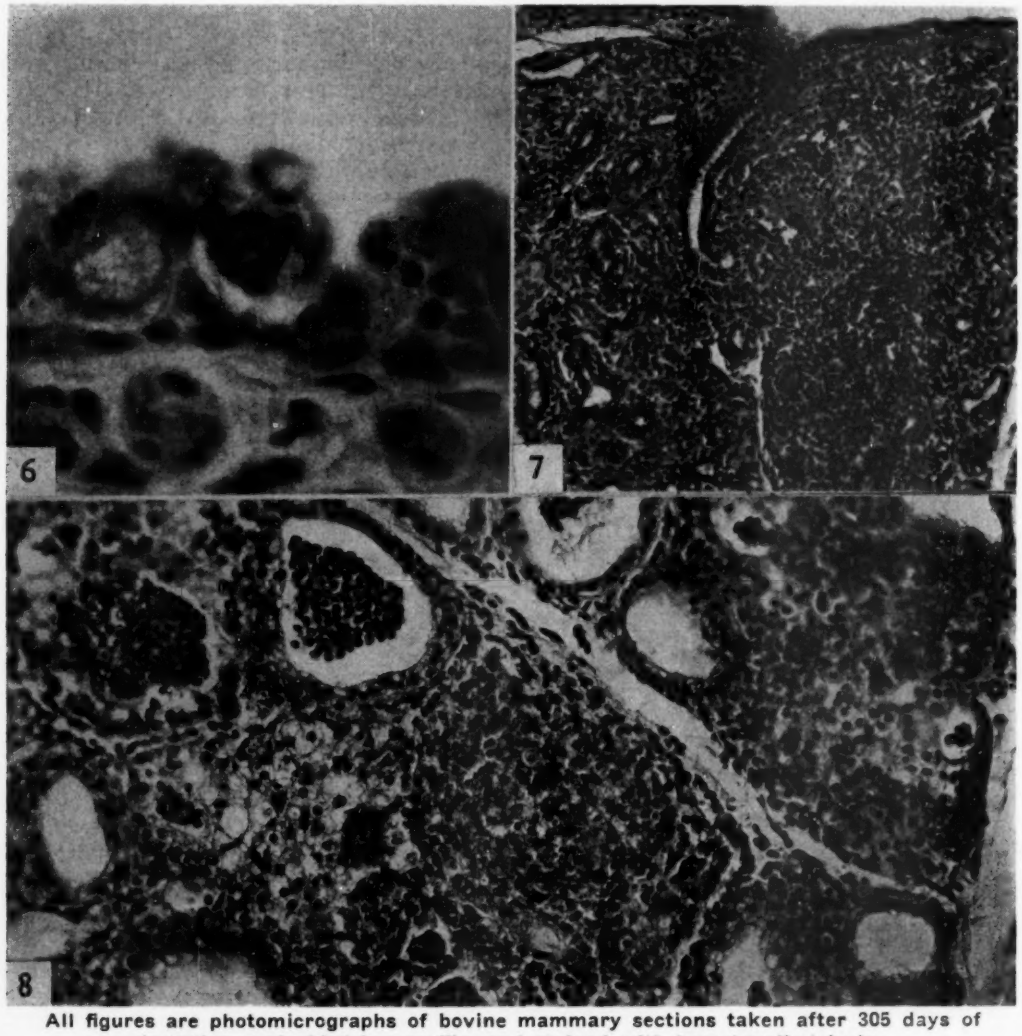
Fig. 4—Section of teat from a Holstein-Friesian heifer milked at 17 in. of vacuum for twice normal duration. Same areas as figure 3. Thelitis consisting of marked lymphocytic infiltration of the lamina propria and the tendency of the lining to form folds. The membranocutaneous is at the right. x 100.

Fig. 5—Lobulus from udder of a Holstein-Friesian heifer milked at 13 in. of vacuum for normal duration. At extreme left colostrum bodies, at top center an early lesion consisting of an alveolus with clotted secretion and a few heterophils, lower center, a more established lesion of atrophied alveoli with interalveolar heterophilic and lymphocytic infiltration are seen. x 105.

that the causative organisms would have easily escaped detection by the usual bacteriological examination.

Involuting lobuli were seen in all udders. As illustrated (fig. 7), they consisted chiefly of atrophying alveoli and still prominent intralobular ducts with lymphocytes in the interalveolar tissue. Such a picture has been considered as mastitis and difficulty has been admitted [21, 29] in distinguishing between mastitis and involution. In view of the continued negative history of udders with such changes and the fact that lactation had continued for 305 days, such changes were dismissed as normal involution.

When the occurrence of histological evidence of mastitis in the experimental halves



All figures are photomicrographs of bovine mammary sections taken after 305 days of lactation, embedded in paraffin, and stained with hematoxylin-triosin.

Fig. 6—Section from same area and heifer as in figure 4. A small corpus amylaceum is seen in the membranous epithelium. x 550.

Fig. 7—Portion of a lobulus from udder of a Holstein-Friesian heifer milked at 10 in. of vacuum for twice normal period. Involution is advanced with alveoli largely absent and only intralobular ducts remaining. There is lymphocytic infiltration of the interalveolar tissue, a normal condition in involuting udders. At top center the double-layered epithelium identifies an interlobular duct. x 100.

Fig. 8—Section of udder from a Holstein-Friesian heifer milked at 17 in. of vacuum at twice normal duration. A focus of established mastitis is shown. In upper left, masses of heterophils are seen in the alveoli, while in lower center the alveoli have atrophied and the leukocytes are seen to be established in the interalveolar tissue. x 165.

was tested by *chi* square,[13] no significent differences among vacuum levels was noted. The normal halves had a higher occurrence of mastitis alterations and the twice normal halves a lower occurrence than could reasonably be expected ($P < 0.10$) by chance.

The occurrence of thelitis as evidence of lymphocytic infiltration of the lamina propia or hyperplasia showed no significant difference among vacuum levels or between milking durations when tested similarly.

Supramammary Lymph Nodes. — All lymph nodes were considered normal when examined histologically. Gross observations which consisted of weight and measurements were likewise insignificant.

SUMMARY

Eleven first-calf heifers were considered to be mastitis-free after a thirty-day postpartum period of twice weekly studies which consisted of leukocyte count, pH and chloride value, and bacteriological examination of the milk. The animals were then divided at random into three groups according to inches of vacuum of the milking machine; these were 10, 13, and 17 in. One half of each udder was randomly assigned to a normal duration of milking while the other half was milked for the twice normal duration. At 305 days postpartum, animals were slaughtered and histological sections were taken from the udders and other tissue. Histological changes of focal acute mastitis and thelitis were seen and occurred without relation to vacuum of milk machine or duration of milking. The acute foci of mastitis did not appear to result in fibrosis but rather in involution of the involved areas, which process was considered to be a defense mechanism. Log of the leukocyte count, chloride and pH values, and freedom from mastitis organisms showed no signifiicant differences in average response among vacuum levels. The same applied for duration of milking time with the exception that there was a significant greater mean leukocyte and chloride value for the halves milked at the normal duration.

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Retained Placenta—Experimental Production and Prevention

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RETENTION of the placenta by the cow following parturition is a major disease problem.

Kennedy [1] reported an incidence of placental retentions of 8.3 per cent of 395 deliveries in English herds where brucellosis was absent or "quiescent." Palmer [2] recorded the retention of the fetal membranes in 11.7 per cent of 125 cows in two herds which were free of brucellosis and tuberculosis. Boley [3] cited a herd of 200 cows, free of brucellosis, which had had *Vibrio fetus* isolated from an aborted fetus three years earlier, wherein retentions occurred following 12.5 per cent of the deliveries. Boyd and Sellers [4] observed that 6.4 per cent of 450 parturitions were followed by placental retentions when brucellosis-free cows were selected to include only deliveries of viable single calves.

The etiology of placental retention can be ascribed to infectious agents in some cases. Fincher [5] found about one third of 207 cases of placental retention to occur in cows infected with brucellosis. Smith [6] recorded a high percentage of retentions in cows delivering or aborting while infected with *Vibrio fetus*. The etiology is still obscure in many cases. Kennedy [1] made a histological study of the maternal-fetal placental attachment of 24 cases of retained placentas and found leukocytic infiltration in 1 case. Hart *et al.* [7] and Ronning *et al.* [8] reported up to 69 per cent of the cows had placental retentions when brucellosis-free cows were maintained on a vitamin A deficient diet. Jordan [9] believes placental retention is associated with lack of uterine motility after delivery, whereas Kennedy [1] does not believe that uterine motility affects placental expulsion.

This paper records the findings regarding placental retentions in experimental cows and the prevention of placental retentions in similar experimental animals by progesterone administration prior to delivery.

MATERIALS AND METHODS

This study involved 13 multi- and primaparous Holstein-Friesian cows which were negative to blood serum-agglutination tests for brucellosis. The husbandry of the cows and the technique of removal of the corpus luteum has been discussed in a previous publication.[10]

The pregnancies of group 1 (chart 1) were maintained after corpus luteum ablation by the daily administration of 100 mg. (75 mg. to cow 037) of crystalline progesterone in sesame oil. These injections were discontinued between the 162nd and 237th days of pregnancy without abortion ensuing.

Cows 453 and 454 in group 2 (chart 2) received daily injections of crystalline progesterone in sesame oil after removal of the corpora lutea until days 168 and 184 when the injections were discontinued. Cows 163, 169, and 161 were subjected to removal of the corpus luteum on days 207, 224, and 230, respectively, and did not receive progesterone replacement therapy immediately following corpus ablation. All 5 cows in group 2 received daily injections of 100 mg. of crystalline progesterone in sesame oil for fourteen to thirty days prior to day 278 of the gestation period, which is the expected day of delivery for Holstein-Friesian cows.[11]

RESULTS

Seven of 8 cows in group 1 retained the placenta until seventy-two hours after delivery when manual removal was employed. The remaining cow (037) expelled the placenta promptly after delivery. The average gestation length for the 8 cows was 267.5 days, with a range of 254 to 282. The calves delivered as early as days 254, 258, and 263 were alive but weak at birth while the remaining calves were alive and healthy at delivery. A biopsy of the cotyledon-caruncle attachment was taken sixteen

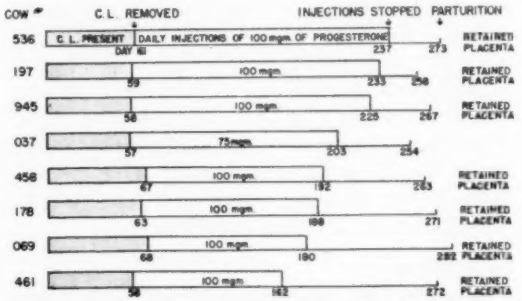

Chart 1—Retained placentas occurred in 7 of 8 cows. The corpora lutea were removed and no progesterone was administered for at least twenty-five days prior to parturition.

Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Paper from the Department of Veterinary Science No. NS 160, University of Wisconsin, Madison.

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hours after delivery from cow 461. A histological examination of sections from this attachment revealed a marked adherence of cotyledonary epithelium to caruncle epi-

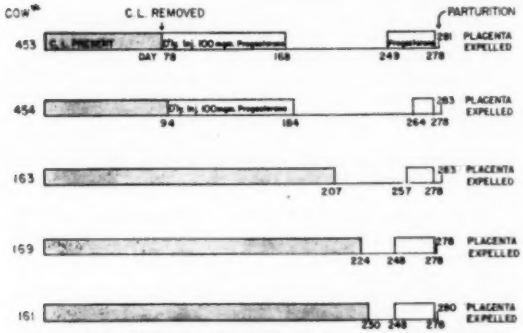

Chart 2—Placenta expelled by 5 of 5 cows in which progesterone replacement therapy was practiced near the end of gestation.

thelium, but leukocytic infiltration or other signs of an inflammatory process were not observed.

Five of 5 cows in group 2 expelled their placentas within twenty-four hours after delivery of live healthy calves. The average delivery date was day 281, with a range of 278 to 283. A biopsy of the cotyledon-caruncle attachment of cow 453 was taken approximately thirty minutes after delivery. A histological examination of sections from this attachment revealed a definite loosening of the cotyledonary epithelium from the caruncular epithelium. There were no signs of placentitis.

DISCUSSION

A statistical analysis of the occurrence of retained placentas in the two groups shows a highly significant difference to exist. The retentions in group 1 may have been due to a lowered level of progesterone or to the abnormally short period of gestation. Six of 8 cows delivered earlier than one standard deviation (4.8 days) before the expected delivery day of 278.[11] One cow (069) delivered four days after the expected delivery date.

The administration of progesterone to the cows in group 2 apparently affected the delivery dates, since none delivered while receiving the injections and the average gestation period was 281 days as contrasted to 267.5 days for group 1.

All 5 cows delivered within one standard deviation of the expected calving date. It is impossible to determine whether the apparent prevention of placental retention in group 2 was due directly to the progesterone level being raised, or indirectly by the resultant inhibition of early delivery.

The absence of leukocytic infiltration and other inflammatory signs in the placental attachment of cow 461 corroborates Kennedy's findings [1] and indicates that retention may occur in the absence of infectious agents and placentitis, such as Hallman [12] found when retention followed brucella infection.

These data suggest a possible role of progesterone in an hormone-enzyme relationship involving the loosening of the placental attachment, as well as its effect on the onset of uterine activity and subsequent delivery. The relationship of some field cases of retained placentas to these experimental cases needs further investigation.

This possible association of low progesterone level with placental retention and early calving leads to the consideration that placental retention frequently is associated with field cases of abortion. Some field cases of abortion may be due to a progesterone deficiency.

SUMMARY

Eight cows were subjected to corpus luteum removal at approximately the sixtieth day of pregnancy, and the pregnancy was maintained by injecting progesterone until the 162nd to 237th day of pregnancy. Upon delivery, 7 of these 8 cows had retentions of the placentas and many had abnormal lengths of gestation periods. Histological study of the cotyledon-caruncle attachment of 1 cow did not reveal signs of placentitis, although there was a firm adherence of the cotyledonary epithelium to caruncular epithelium.

A deficiency of progesterone prior to delivery was suspected as being responsible for the placental retentions, and the experiment was designed to supplement like animals with exogenous progesterone during the last two to four weeks of pregnancy. Five cows were subjected to the removal of the corpus luteum between the seventy-eighth and 230th days of pregnancy and either received no progesterone injections after the removal or only until the 168th or 184th days of pregnancy. Administration of progesterone was begun on the

248th to the 264th day of pregnancy and continued until the 278th day of pregnancy. All 5 cows delivered within one to five days after cessation of injections and all expelled the placentas within twenty-four hours. Histological study of sections of the cotyledon-carunele attachment of 1 cow taken as early as thirty minutes after delivery showed a loosening of the cotyledon-carunele epithelium.

A highly significant difference exists between the two groups relative to the incidence of placental retentions.

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Studies on Intravenous Administration of Calcium, Potassium, and Magnesium to Dairy Calves. II. Some Cardiac and Respiratory Effects

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IN AN EARLIER report, some biochemical and general toxic effects due to intravenous administration of calcium, potassium, and magnesium salts, and sodium oxalate were described.[2] The present paper deals with simultaneous cardiac and respiratory effects of these same administrations. These effects are correlated with the simultaneous blood level of the corresponding ion. Methods are described for restraint of the animal used and for recording of these aforementioned measurements.

METHODS

Figure 1 shows the general experimental procedure used. The calves were restrained in a specially constructed modification of a Pavlov-type dog sling. Two wide heavy canvas belts with four holes for the calf's legs were placed around the body so the animal could not completely lie down and disengage the recording apparatus. The head was restrained by closing the stanchion.

A constant infusion mercury pump was used for injection of the desired solution (fig. 1). The solution to be injected was placed in the lower flask which was connected to a polyethylene tube after cannulation of the jugular vein with a 14-gauge needle. Mercury was placed in the upper flask and its flow through a capillary tip was calibrated so that the solution was injected intravenously at a known rate. These injections were continued to the point of severe symptoms or death.[2]

The concentration, cumulative dose per kilogram of body weight, and duration and rate of injection of the solution studied, together with methods of collection and analysis of blood specimens, have been previously described.[2]

Electrocardiograms were recorded at numerous intervals throughout all of the experiments by utilizing one channel of a twin-channel recorder* (fig. 1). Only precordial leads (apex of heart to top of right scapula) were recorded in view of the fact that this bipolar lead in cattle appears to give the most standard and duplicable results from animal to animal.[19] Measurements of the electrocardiographic records were made as described by Burch and Winsor[4] and by Carter.[7] Pneumograms and phonocardiograms were alternately recorded on the second channel of the twin-channel recorder in the majority of experiments.

In some experiments, right intraventricular pressures were recorded electromanometrically by a modification of the method of Sellers and Hemingway,[20] using the single-channel recorder.† Intraventricular pressures were recorded in an effort to correlate mechanical and volume-pressure relationships of ventricular beats with electrocardiographic changes. If myocardial toxicity occurred as a result of injection of various ions, then effects upon intraventricular pressure should be manifested. Right intraventricular pressure was selected for measurement rather than left intraventricular pressure because the right ventricle is more accessible to cannulation.

The electromanometer was set at a common level with the sternum in view of the fact that this was a readily ascertainable reference point. Corrections for this so-called "zero" were later made by measuring the distance from the middle of the cavity of the right ventricle to the outside of the sternum in 10 calves of a similar weight and age at autopsy. The average distance found was 11 cm. which corresponds to approximately 8 mm. of mercury. This value was subtracted from the recorded blood pressure. Table 2 includes average values of this "corrected" blood pressure. Figures 3 and 6 show recorded blood pressures.

A cardiac catheter‡ was inserted into the jugular vein by means of a trocar and cannula (12 cm. in length and 0.4 cm. in diameter) and, thence, downward into the right ventricle. Local anesthesia was produced by infiltrating the area with 10 to 15 ml. of 2 per cent procaine hydrochloride.

If the animal was standing, no difficulty of bypassing the heart and entering the posterior vena cava was encountered; if the animal was being completely supported by the sling, considerable difficulty was at times encountered, presumably due to alterations in the normal position of the heart. One could readily tell when the catheter

Approved for publication as scientific journal series paper No. 2996, Minnesota Agricultural Experiment Station.

This investigation was made possible through a generous grant from the Greater University Fund, University of Minnesota, St. Paul.

Data taken in part from a thesis to be submitted by the senior author to the graduate school, University of Minnesota, in partial fulfillment of the requirements for the degree of master of science. The authors wish to express their appreciation to Mr. A. O. Stensrud for his help in the progress of this study and to Dr. J. H. Sautter for taking some of the photographs.

* Hot wire direct-writing galvanometer. Sanborn Co., Cambridge, Mass.

† Hot wire direct-writing galvanometer. Sanborn Co., Cambridge, Mass.

‡ Cardiac catheter 115 cm. in length, 0.15 cm. inside diameter. U. S. Catheter and Instrument Corp., Glens Falls, N. Y.

tip went through the right atrioventricular orifice by observing concomitant blood pressure changes on the recording system. Measuring the approximated distance between the point of cannulation of the vein and the heart on the catheter also seemed to be of help in establishing the location of the atrioventricular valve. The middle of the ventricle was then approximated by advancing the catheter tip about 4 cm. beyond the atrioventricular valve, and the catheter tip left in this position. Figure 2 shows the position of the cannula in the middle of the right ventricle in an animal autopsied after a typical fatal experiment. Adhesive tape and gauze were placed around the calf's neck over the point of entrance into the jugular vein. In this way, bleeding was stopped and the catheter immobilized. Only a slight hematoma usually developed and several cannulations were possible in the same animal within a period of a few days.

A 500-ml. bottle of heparinized 0.9 per cent sodium chloride solution was placed above and connected to the heart catheter by a three-way stopcock to give a continuous slow drip, and thus prevent coagulation of blood between recordings. Plasma sodium was measured in only those experiments where no saline solution was injected.

A dual marker switch was installed on the single- and twin-channel recorders so that respiratory movements, blood pressure, and electrocardiographic records could all be simultaneously recorded and aligned with respect to time.

RESULTS

Tables 1 and 2 give average data for electrocardiographic effects, respiratory movements, and right ventricular pressures before and immediately after cessation of injections for all substances used. Typical records are shown in figures 3 through 6.

Calcium Administrations.—When calcium was given intravenously, very striking effects were observed (fig. 3 and 4). Gluconate and chloride salts seemed to elicit the same type of response but effects of the latter salt appeared to be more severe and were delayed.[2] A marked bradycardia was always associated with these experiments. As the injection was continued, various grades of heart block and isolated or small groups of extrasystoles interrupted this slowed cardiac rhythm. Systolic sounds of markedly increased amplitude and duration were frequently recorded phonocardiographically. At vari-

Fig. 1—General experimental procedure used. Constant infusion mercury pump at upper left; twin-channel recorder for recording pneumogram, phonocardiogram, and electrocardiogram at left center; electromanometer and single-channel recorder for blood pressure measurement on table at right.

ous unpredictable times, short periods of nodal (or ventricular) escape and tachycardia occurred. Death followed ventricular fibrillation, which event would suddenly appear following such a phase of ventricular tachycardia. Three deaths resulted in a total of nine calcium experiments. Two of these animals died immediately of ventricular fibrillation during injection of calcium chloride. The third animal died approximately four hours after presumed recovery.[2] Two other animals exhibited a temporary atrial flutter.

A marked rise in right ventricular systolic pressure was observed in these calcium experiments. The pressure exhibited per beat seemed to be roughly proportional to the amplitude of the QRS* complex. In one experiment which included carotid pressure measurement (fig. 4), the mean pressure did not appear to change, although the pulse pressure increased. When fibrillation occurred, the blood pressure became markedly reduced. Respiratory movements were increased in rate and amplitude (table 1).

* P, Q, R, S, and T are designations of waves in electrocardiograms.

In all of the calcium experiments, slowing of intracardiac conduction became more marked as injections were continued (table 2). Atrioventricular conduction, as measured by the P-R interval, appeared to be the most markedly prolonged. Various degrees of atrioventricular block occurred, but partial heart block of 3:2 or 4:3 grade seemed to be the most common. In these instances, the P-R interval progressively increased with each successive beat until it became so prolonged that no ventricular complex occurred (Wenckebach phenomenon). Intraventricular conduction, as measured by the duration of QRS, was sometimes slowed but to a lesser degree than atrioventricular conduction. In one experiment (fig. 4), a record was obtained that resembled intraventricular block of the bundle branch type.[7] In this record, there is notching and prolongation of the QRS complex.

The duration of electrical systole, as measured by the Q-T interval, was shortened in spite of the fact that the heart rate and intracardiac conduction were both slowed. Amplitudes of QRS and T waves, indicating electrical potential, increased in

TABLE 1—Circulatory and Respiratory Changes (Average) Before and After Intravenous Injection of Several Ions to the Point of Severe Symptoms or Death[1]

No. of exps.	Substance injected[2]	Average blood level mEq./liter[3]		Rate per minute				Right ventricular pressure[6] (millimeters of mercury)				General electrocardiographic effects
				heart rate		resp. rate[5]		diastolic		systolic		
		before	after	before	after	before	after	before	after	before	after	
9	Calcium	5.1	12.8	109	65 (early) 113 (late)[4]	26	48	0	3	35	70	Marked bradycardia, then various degrees of heart block with extrasystoles and terminal fibrillation. Atrial flutter in 2 experiments.
8	Potassium	4.5	8.6	107	121 (early) 49 (late)	30	44	....	....	....	....	Progressive intraventricular block. Atrial flutter and heart block in 2 experiments. Temporary ventricular arrest in 1 exp. Occasional S-T* depression.
5	Magnesium	1.9	11.9	97	96	38	48	7	8	57	50	Slight S-T slur.
2	Magnesium + Calcium	2.2 Mg 5.4 Ca	12.9 Mg 15.9 Ca	130	163[4]	58	42	2	1	44	55	Tachycardia. Occasional heart block and extrasystoles. S-T slur.
2	Na Oxalate	4.6 Ca	2.4 Ca	103	131	32	82	2	7	54	54	S-T slur.

[1] Fatal experiments included: 3 of calcium, 1 of potassium, 1 of magnesium, and 1 of magnesium and calcium together. All values are averages taken before injections and immediately after cessation of injections.

[2] All cations were injected as the chloride salts with the exception of 2 experiments using calcium gluconate.

[3] Total serum calcium, plasma potassium, and plasma magnesium.

[4] Not including terminal tachycardia.

[5] Based on only 5 calcium and 4 potassium experiments and 1 magnesium experiment.

[6] Based on only 4 calcium experiments and 1 magnesium experiment. Right ventricular pressure became constant at 20 to 25 mm. Hg in those experiments where fibrillation occurred.

* Designation of wave on electrocardiogram.

TABLE 2—Average Values of Components of Electrocardiogram Before and After Intravenous Injection of Several Ions to the Point of Severe Symptoms or Death[1]

No. of exps.	Substance injected	Average blood level mEq./liter		Time in seconds						Amplitude in millivolts					
				P-R*		QRS		Q-T		T		P		QRS		T	
		before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
9	Calcium	5.1	12.8	0.13	0.21	0.07	0.08	0.30	0.24	0.10	0.13	0.21	0.22	1.31	1.61	0.35	0.64
7	Potassium	4.6	8.1	0.13	0.11	0.07	0.08	0.29	0.33	0.11	0.08	0.18	0.0	1.61	1.76	0.46	0.93
1**	Potassium	4.0	12.7	0.13	.....	0.06	0.20	0.32	0.52	0.06	0.36	0.15	0.0	1.10	0.80	0.10	1.30
5	Magnesium	1.9	11.9	0.15	0.18	0.07	0.09	0.30	0.32	0.12	0.14	0.23	0.20	1.50	1.75	0.52	0.71
2	Magnesium	2.2 Mg	12.9 Mg	0.12	0.22	0.06	0.10	0.25	0.22	0.10	0.14	0.18	0.23	1.13	1.60	0.43	1.30
	+ Calcium	5.4 Ca	15.9 Ca														
2	Na Oxalate	4.6 Ca	2.4 Ca	0.13	0.10	0.06	0.06	0.27	0.33	0.10	0.15	0.25	0.25	1.25	1.95	0.35	1.05

[1] See footnotes, table 1.
* P, Q, R, S, T are designations of waves on the electrocardiogram. ** Fatal experiment. Greater differences were observed than in the other experiments.

all of the calcium injections. These amplitudes decreased at the terminal stage (tachycardia or ventricular fibrillation), however. Occasional notching of P waves was seen.

Potassium Administrations.—Injection of potassium was without regular effect on heart rate, until potassium levels in the plasma of 8 or more mEq./liter were attained. At this level, the rate became progressively slower. Respiratory movements increased in rate and amplitude in all potassium experiments. Atrial flutter and complete atrioventricular block with nodal rhythm was observed in two of eight experiments, and temporary ventricular arrest of eight seconds' duration was noted in another experiment. No ventricular extrasystoles were noted. One death occurred at a plasma potassium level of 12.7 mEq./liter.[2] This animal died, presumably due to a progressive cardiac arrest (fig. 5). The heart rate was 43/minute just prior to death.

No marked changes in intracardiac conduction were noted with potassium injection until potassium levels in the plasma of approximately 8 mEq./liter were attained. Below this level, the P-R interval was shortened but the QRS duration was slightly prolonged (table 2). At potassium levels higher than 8 mEq./liter, however, a progressive and very marked prolongation of intracardiac conduction time occurred. Complete disorganization of the QRS complex and T wave occurred, indicating widespread intraventricular block.[5,6,7] The peculiar notching of the QRS complex in one experiment (fig. 5) resembles intraventricular block of bundle branch type.[6,7] All P waves became progressively reduced in amplitude and completely disappeared at potassium levels of 7 to 8 mEq./liter in the plasma; thus, the P-R interval could not be measured.

The duration of electrical systole (Q-T interval) became progressively and very markedly prolonged as the potassium level in the plasma rose from a normal value of 5 mEq./liter to the lethal level of 12.7 mEq./liter. The amplitude and duration of the T wave also markedly increased (table 2). In one experiment, a depression of the S-T segment was observed on alternate beats. When potassium injections were stopped, a progressive recovery oc-

curred and no permanent cardiac damage was observed when these animals were tested electrocardiographically prior to subsequent experiments.

Magnesium Administrations.—No marked change in heart rate was observed with magnesium injection. No arrhythmias or ectopic beats were noted. Respiratory movements and right ventricular pressure were recorded in only one of five magnesium injections. In this experiment, respiration increased in rate and amplitude and systolic pressure decreased slightly (table 1).

All magnesium injections were carried to the point of light anesthesia. One death occurred following respiratory failure. A rapid rate of injection of magnesium chloride had been given in this experiment.[2]

Magnesium injection slowed all phases of intracardiac conduction. Atrioventricular conduction (P-R interval), together with intraventricular conduction (QRS duration), was depressed (table 2). The duration of electrical systole (Q-T interval) was only slightly prolonged. When injection of magnesium was stopped, a progressive recovery occured and no permanent damage was observed.

Simultaneous Magnesium and Calcium Administrations.—When magnesium and calcium chloride were given simultaneously to 2 different animals, the heart rate progressively increased so that a marked sinus tachycardia existed at the end of the injection (table 1). In one experiment, a partial atrioventricular block with the Wenckebach phenomenon present, and numerous isolated extrasystoles was superimposed upon this rapid cardiac rate. In the other experiment (fig. 6), no extrasystoles were observed but anesthesia was seen to be followed by ventricular fibrillation and death. This animal presumably died from the toxic effects of calcium.[2] Tables 1 and 2 give the toxic blood levels of these ions.

Systolic sounds of increased duration and amplitude were frequently recorded when magnesium and calcium were given together. Respiratory movements decreased in rate and amplitude and right ventricular systolic pressure moderately increased (table 1). These two ions appeared to have antagonistic effects in this respect.

Fig. 2—Autopsy of calf after a typical experiment. Apical and cardiac lobes of lung were removed and right ventricle opened to show position of catheter in middle of ventricle.

Atrioventricular and intraventricular conduction and ventricular repolarization (P-R, QRS, and T durations, respectively) were markedly prolonged (table 2). The effects of magnesium and calcium seemed to be additive in this respect. The duration of electrical systole (Q-T interval) was shortened slightly. The amplitudes of the various waves were markedly increased. The P wave, however, was usually obscured by the preceding T wave because of the sinus tachycardia exhibited and thus was difficult to measure.

Sodium Oxalate Administrations.—When hypocalcemia was induced in 2 animals, no cardiac arrhythmias or ectopic beats were observed. The heart rate, however, moderately increased. The respiratory rate was very markedly increased in both experiments. No marked change in right ventricular pressure was seen (table 1).

Atrioventricular conduction time (P-R interval) was shortened with oxalate injection, but no marked change in intraventricular conduction (QRS duration) was observed. Like potassium injection, oxalate injection produced a lengthening of electrical systole, and a marked elevation of the QRS and T waves. The T wave increased in duration to a degree somewhat more marked than in the other types of experiments (table 2).

An elevation in level of plasma potassium occurred in both sodium oxalate experiments. Possible sodium interference resulting in erroneously high potassium determinations in these sodium oxalate injection experiments was considered. Additions of sodium oxalate to bovine plasma *in vitro* were made, using increments of approximately 3 mEq./liter, from 139 to 152 mEq. of plasma sodium/liter. Under these conditions of *in vitro* addition, replicate determinations of potassium, in the presence of added sodium as noted, were within the limits of experimental error. It was felt, therefore, that the elevation of plasma potassium observed *in vivo* when sodium oxalate was injected was probably a true physiological response. This may have been contributory to the development of some of the effects that were similar to potassium injection.

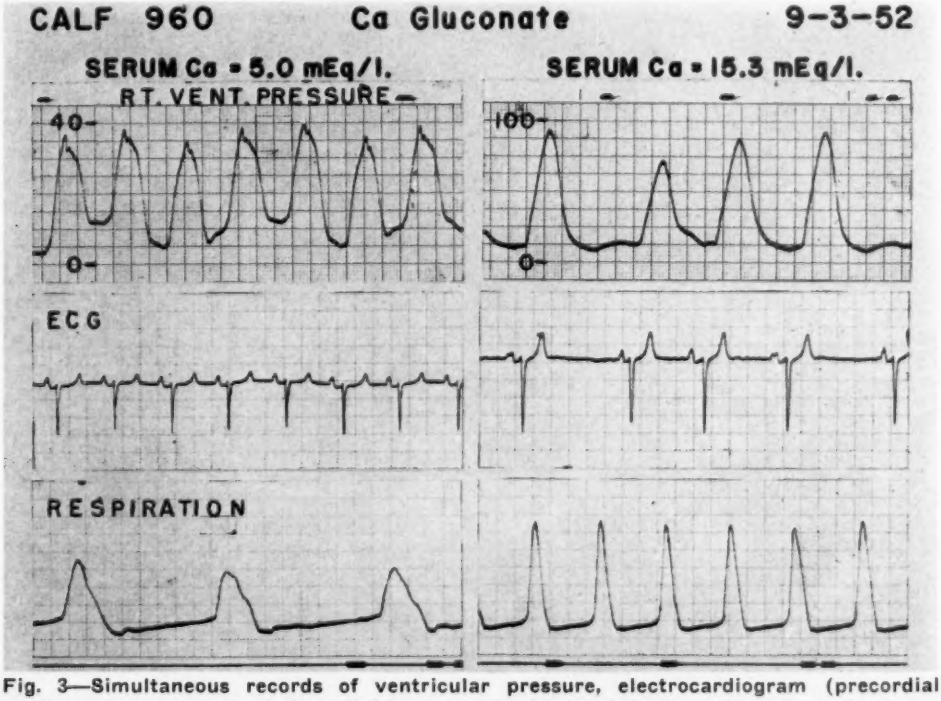

Fig. 3—Simultaneous records of ventricular pressure, electrocardiogram (precordial lead), and pneumogram before (left), and after (right) injection of calcium gluconate. Pressures are in millimeters of mercury. Note marked increase in systolic pressure, and bradycardia in tracings on right.

DISCUSSION

Calcium Administrations.—An initial marked bradycardia, but terminal tachycardia and/or fibrillation, was associated with intravenous calcium administration. These results are in agreement with experiments on other animals.[8, 15-17, 29] In dogs, the initial bradycardia has been attributed to vagus stimulation since it is abolished by atropine; the second phase, *i.e.*, tachycardia, is potentiated by atropine and, therefore, is said to be due to a sympathetic effect or to a direct stimulating effect on the myocardium.[15, 16, 29]

In the present calcium experiments, the decrease in automaticity of the sino-atrial node, together with slowing of atrioventricular conduction, appeared to initiate the occurrence of partial heart block with numerous isolated ventricular extrasystoles (fig. 4), presumably arising from a single ectopic focus within the ventricles. The periods of ventricular tachycardia (ventricular escape) that subsequently developed may have originated from single or multiple ectopic foci.[4, 7]

It appears, then, that intracardiac automaticity was not uniformly affected over the entire heart, in view of the fact that foci of preserved activity seemed to persist and bring about these extrasystoles, and also the fact that local blocks in conduction later seemed to develop (atrioventricular block, notching, and widening of QRS). These local blocks, multiple foci of preserved activity, and slowed intracardiac conduction would seem to be of importance in the genesis of the atrial or ventricular fibrillation observed.[3] Nahum and Hoff [18] state that the essential condition for onset of fibrillation is the establishment of blocks of the conducting system in a heart whose automaticity is simultaneously stimulated: either factor alone is ineffective. Calcium appeared to fulfill these conditions in its second phase of action.

Right ventricular systolic pressure rose markedly with calcium administration.

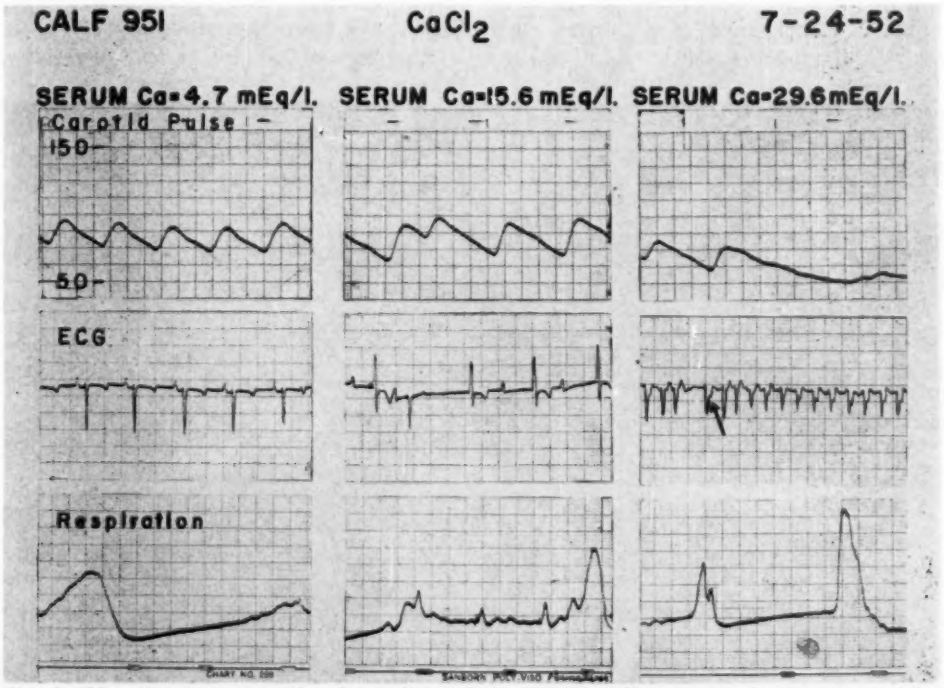

Fig. 4—Simultaneous records of carotid pulse, electrocardiogram, and pneumogram before (left), during (center), and after (right), an injection of calcium chloride. Note heart block and extrasystoles in center record and ventricular tachycardia with sudden fall in carotid pressure in right record; a few seconds later, typical ventricular fibrillation resembling that in figure 6 was seen, followed by death of the animal. Arrow indicates probable bundle branch block.

This rise presumably may indicate an increased and compensatory ventricular filling and ejection of blood and/or increased contractile force of the myocardium. Effects on the pulmonary peripheral resistance could also conceivably result in a rise in right intraventricular pressure. The increase in carotid pulse pressure with little change in mean carotid pressure in one experiment is in agreement with experiments on human beings [8] and dogs.[17]

The duration of the Q-T interval was shortened with calcium injection; this interval according to present concepts is understood to be the duration of electrical systole but not necessarily of mechanical systole.[4, 7, 30] The S-T interval (duration of depolarized state) underwent the greatest change since both QRS and T durations were lengthened.

Potassium Administrations.—The action of potassium on the heart was different from that of calcium. Marked elevation of T waves and gradual disappearance of P waves were initial effects observed with potassium injections. According to present concepts, disappearance of P waves does not necessarily mean cessation of atrial activity,[5, 6] although atrial cessation after potassium injection has been occasionally observed in the exposed dog heart.[27]

Widespread intraventricular block with occasional nodal rhythm and gradual cardiac arrest were noted after potassium levels of 8 mEq./liter were attained in the plasma. This decrease in automaticity appeared to have been uniformly produced over the entirety of the ventricles; this was in contrast to the action of calcium which seemed to produce areas of local block, foci of preserved activity, and later a second phase of increased automaticity ending in ventricular fibrillation. Various investigators [5, 6, 9, 18, 27–29] have reported similar observations in other animals.

Nahum and Hoff [18] state that when potassium was slowly injected in cats and rabbits, the automaticity of the pacemaker and of other cardiac structures is gradually reduced to zero but, when injected rapidly, a stimulation of automaticity occurs. If intraventricular block is present when automaticity is thus stimulated, fibrillation will result. These latter factors may have been the cause of the occasional temporary atrial flutter that was observed

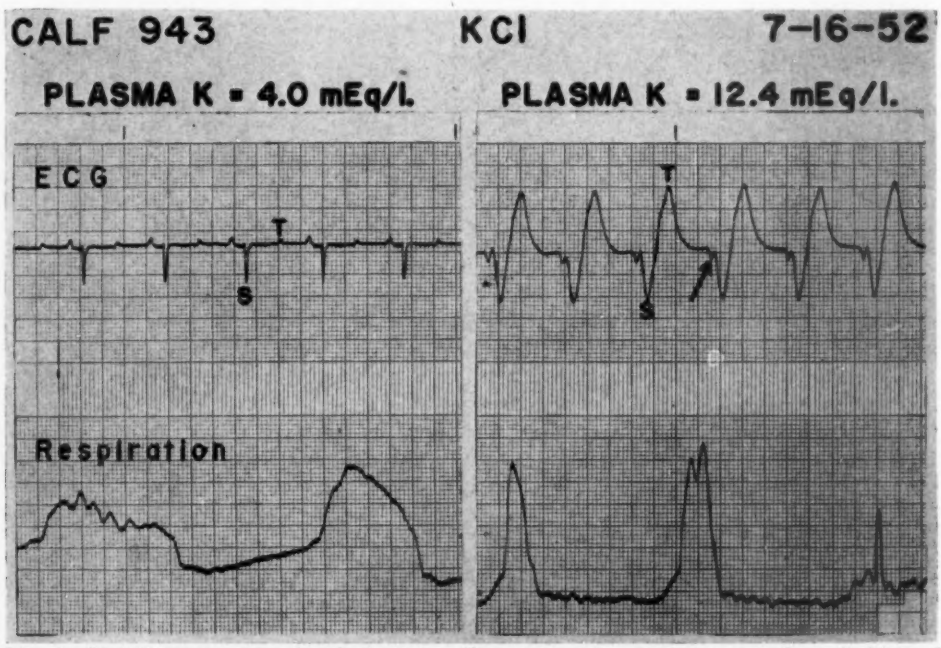

Fig. 5—Simultaneous records of electrocardiograms and pneumogram before (left) and after (right) injection of potassium chloride. Note marked widening of QRS and T wave (widespread intraventricular block), and absence of P waves. Arrow indicates probable bundle branch block.

in the present experiments with dairy calves.

Burchell [5,6] and others [18,27] report that potassium injection in dogs produces a gross intraventricular block which superficially resembles right bundle branch block. In one of the present experiments (fig. 5), records were obtained that show a striking resemblance to those obtained in dogs by the above investigators. Also, in one calcium experiment (fig. 4), records were obtained that resembled block of the opposite (left) His's bundle. Alfredson and Sykes,[1] however, reported that cattle differ from dogs in their distribution of intraventricular conducting tissue and that when one His's bundle of the bovine heart is severed, little or no change in the electrocardiogram will occur.

High levels of plasma potassium also produced a marked lengthening of the Q-T interval, indicating a prolongation of electrical systole. An interesting fact is that hypopotassemia has also prolonged the Q-T interval in dogs [5] and in cattle.[26] The occasional S-T depression observed with the present potassium injections tends to indicate that large regions of the ventricles fail to contribute their usual electrical stresses because of injury.[4,7]

Magnesium Administrations. — Magnesium appeared to cause death from respiratory failure after the attainment of anesthesia. The effects upon the cardiovascular system appeared not to be marked. This result is in agreement with experiments on dogs, cats, and rabbits, as reported by other investigators.[11,17,21,22,29] These authors also state that severe cardiac disturbances result only if very high blood levels of magnesium are attained while artificial respiration is geing given.

The action of magnesium on the bovine heart differed from that of calcium and potassium in that both atrioventricular and intraventricular conduction were depressed. Calcium seemed to primarily depress atrioventricular conduction and affect cardiac automaticity in an irregular manner. Potassium seemed to markedly and uniformly depress intraventricular conduction as well as cardiac automaticity. Since no extrasystoles were observed with magnesium administration, it would appear that depression of intracardiac conduction was uniformly produced over the

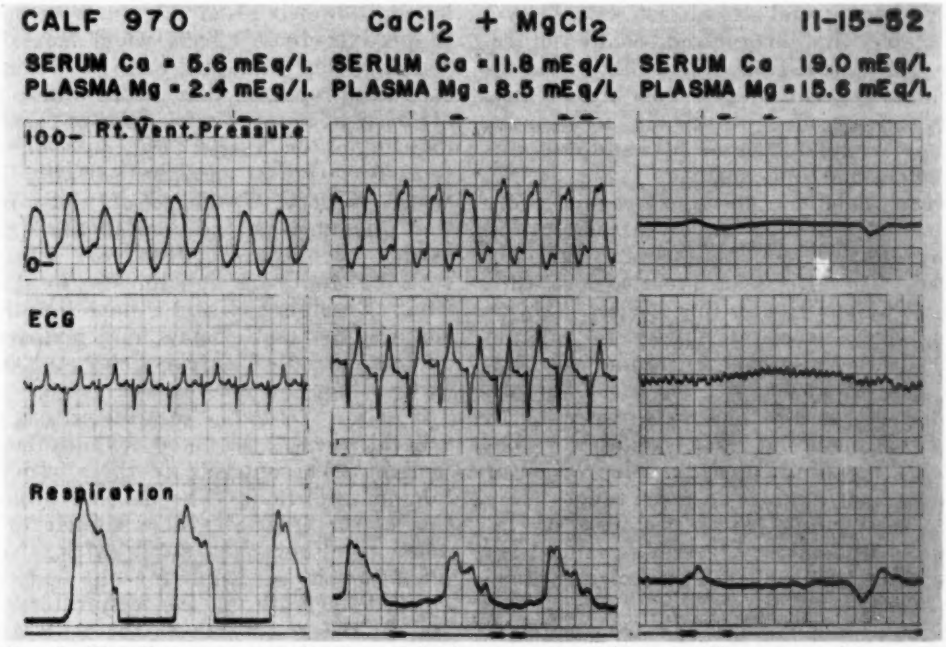

Fig. 6—Simultaneous records of ventricular pressure, electrocardiogram, and pneumogram before (left), during (center), and after (right) injection of a mixture of the chlorides of calcium and magnesium. Note tachycardia in center record and ventricular fibrillation in record at right.

entire heart.[4,7] Prolongation of the Q-T interval was prolonged due to the simultaneous increase in QRS and T wave durations.

In one magnesium experiment, the right ventricular systolic pressure was slightly reduced even though a normal cardiac rate was maintained. Stanbury and Farah [24,25] report that with experimental hypermagnesia in the dog heart-lung preparation, the heart rate and systemic output progressively decrease but that the stroke volume increased. The slowing of the heart rate in their experiments was attributed to a blocking of the cardioaccelerator ganglia and also to a direct depressant action on the sino-atrial node. The experimental procedure and handling of the animal could very well have prevented a slowing of the heart rate in the present experiments with dairy calves.

Simultaneous Magnesium and Calcium Administrations.—An interesting observation was that no initial bradycardia resulted but instead the heart rate progressively increased when calcium and magnesium were injected simultaneously. Calcium, when injected alone, produced a marked bradycardia which has been attributed to a vagal stimulation. Stanbury [24] has stated that magnesium could not release the heart from vagal inhibition in decentralized cat hearts, possibly because of its slowing effect on the sino-atrial node. Thus, no explanation is at hand for this aforementioned phenomenon.

When calcium and magnesium were given together, both atrioventricular and intraventricular conduction were markedly slowed. The effects of the two ions thus seem to be additive in this respect. Major toxic effects were attributed to calcium since death followed ventricular fibrillation. The incidence of heart block and the number of extrasystoles were reduced. In one experiment, no extrasystoles were observed. It appears then, that depression of intracardiac conduction was much more widespread and that fewer foci of increased automaticity were produced in these experiments than when calcium alone was injected.

Right ventricular systolic pressure moderately increased along with an increase in heart rate when magnesium and calcium were injected together. As previously stated, calcium alone caused a marked increase in right ventricular pressure but magnesium alone caused a slight decrease. Therefore, an antagonistic effect of these two ions seems to be displayed. These results appear to be in accord with those of Stanbury and Farah [25] who showed that calcium improved the output and competence of canine hearts previously rendered inefficient by magnesium.

Respiratory movements increased in rate and amplitude when calcium or magnesium were given separately. Intravenous injection of chlorides of calcium or magnesium may possibly give rise to acidosis. If such an acidity did develop, a state of hyperpnea might be expected. However, respiratory movements decreased in rate and amplitude when chlorides of calcium and magnesium were given together. It is known [11] that calcium counteracts both central and peripheral depressant effects of magnesium. This antagonistic nervous effect may, therefore, partially explain the observed respiratory effects.

It is felt that magnesium contributed very little to a reduction of toxicity due to calcium, since only slightly higher serum calcium levels were required to produce toxic effects when magnesium was also given. Lower doses of calcium were needed to produce these effects when magnesium was injected simultaneously.[2] Other investigators reported that in dogs there is no evidence of antagonistic effects of these two ions upon the heart [22] but, instead, their total toxic effects may be partially additive.[21] In dogs, Winkler *et al.*[28] have found that calcium is partially antagonistic to the cardiac depressant effects of potassium rather than being antagonistic to magnesium. Enselberg *et al.*[12,13] have found that magnesium and potassium temporarily abolish or reduce extrasystoles and tachycardias from various causes.

Sodium Oxalate Administrations.—In general, the effects noted with oxalate injections seem opposite to those seen with calcium injections. The marked prolongation of the Q-T interval is similar to that noted by others [10] in adult cattle. No explanation is at hand for the extremely marked increase in the respiratory rate and in the amplitude of the QRS and T waves. Oxalate injection did not produce any extrasystoles or arrhythmias as reported by Spörri and Raggenbass [23] in observations in a single case of bovine milk

fever. One can not say whether the systemic blood pressure becomes low as the result of these injections as has been reported in cases of milk fever.[14]

SUMMARY

1) Methods of simultaneously recording right intraventricular pressure, electrocardiogram, phonocardiogram, and pneumogram in the dairy calf during a constant and continuous intravenous injection have been described.

2) Calcium injection produced an initial bradycardia but terminal tachycardia and fibrillation followed. Various grades of atrioventricular block and isolated or small groups of extrasystoles were observed. A marked rise in right ventricular systolic pressure was seen.

3) Potassium injection seemed to produce a progressive, uniform, and widespread intraventricular block and also a progressive decline in cardiac automaticity until cardiac arrest occurred. P waves disappeared and intraventricular block was established at a plasma potassium level of approximately 8 mEq./liter.

4) Magnesium injection produced respiratory embarrassment and one respiratory failure. Atrioventricular and intraventricular conduction seemed to be progressively and uniformly depressed.

5) Some antagonistic and additive effects of calcium and magnesium were observed and discussed. Calcium appeared to antagonize the toxic effects of magnesium, but the reciprocal antagonism did not seem to occur.

6) The effects of oxalate administration seemed opposite to that of calcium administration.

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The Survival and Transmission of *Trichomonas Foetus* in Diluted Bovine Semen

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WITH THE increasing importance of artificial breeding of cattle, additional information as to the likelihood of transmission of trichomoniasis is needed.

It has been shown by Garlick[1] and by Bartlett and co-workers[2] that trichomoniasis may be transmitted from infected bulls to susceptible females by artificial insemination with fresh undiluted semen. Vandeplassche and co-workers[3] reported that use of diluted semen from an infected bull apparently resulted in a very low rate of infection in the females bred. Sellers and co-workers[4] found no clinical evidences of infection in 22 heifers from the use of specially processed semen from an infected bull. Joyner and Millar[5] reported that trichomoniasis may be transmitted from 1 bull to another by means of the artificial vagina, and that *Trichomonas foetus* was able to survive for several days at 4 to 8 C. in egg yolk buffer mixture containing streptomycin. Bartlett and co-workers[6] reported that a half-dozen instances of transmission of trichomoniasis by artificial insemination had very probably occurred in several units studied by them, and estimated that the rate of transmission by infected bulls may usually have been less than 1 per cent.

The occurrence of trichomoniasis in a bull at an artificial insemination unit using citrated egg yolk containing sulfanilamide, penicillin, and streptomycin for dilution of semen indicated the need of information as to viability of *T. foetus* in such mixtures, and experiments were carried out to obtain this information. An opportunity to observe the results of use of diluted semen from an infected bull occurred when semen from a bull newly purchased by the unit was used to inseminate 32 females prior to its examination for trichomoniasis.

METHODS

Three series of trials were conducted, in each of which duration of survival of *T. foetus* under conditions used for dilution of semen was determined. In one series of 14 trials, trichomonads from cultures were tested for survival in egg yolk buffer mixtures without semen. In each trial, five vials were set up. Vials 1 and 2 each contained 10 cc. of culture medium. Vial 3 contained 10 cc. of 0.85 per cent sterile saline solution. Vial 4 contained 10 cc. of egg yolk buffer mixture with sulfanilamide, and vial 5 was similar to vial 4 except 500 units of penicillin and 0.5 mg. of streptomycin were added per cubic centimeter of total fluid. In trials 5, 7, and 10 to 14, inclusive, 1,000 units of penicillin and 1 mg. of streptomycin were added per cubic centimeter of total fluid. The egg yolk buffer mixture was made up as it is used for dilution of semen: 75 cc. of sterile distilled water, 25 cc. of egg yolk, 2.9 Gm. of sodium citrate, and 0.6 Gm. of sulfanilamide per 100 cc. of mixture. To each of the vials was added 0.5 cc. of a 3-day-old culture of *T. foetus* containing an average of 4,300,000 organisms per cubic centimeter. The culture medium was similar to that of Plastridge,[7] except that beef extract was used instead of beef infusion and 1 Gm. of sodium chloride was added per liter. The cultures were established from preputial samples within three weeks of the time they were used in this experiment. Vial 1 was kept at room temperature; the remainder were refrigerated at 39 F., except for approximately one hour prior to each daily examination, when they were allowed to warm to room temperature.

At each examination, the fluid in the vial was mixed by tilting the vial back and forth or by gently shaking until all sedimented particles, such as egg yolk, were thoroughly dispersed in the fluid. Then a small portion of the fluid was withdrawn with a flamed wire loop, sterile pipette, or sterile syringe, and was placed on a hemacytometer and the concentration of active trichomonads estimated and recorded. When difficulty in finding trichomonads was encountered, as in samples containing egg yolk, at least 7.8 cmm. of fluid was examined. Daily examinations of each vial were made until trichomonads had apparently disappeared. In the first nine trials, inoculations of culture medium with approximately 0.2 cc. of egg yolk buffer solution from vials 4 and 5 were made after active trichomonads were no longer demonstrable. Beginning with trial 10 inoculations were made daily from vials 4 and 5.

In a second series of three trials a similar procedure was used, except that 0.5 cc. of semen was added to each vial containing egg yolk buffer mixture, and 1,000 units of penicillin and 1 mg. of streptomycin was added to vial 5 in each trial. Because of the activity of the sperm in samples from these vials, it was impossible to determine accurately the presence of trichomonads by direct examination. Therefore in this series of trials, samples from these vials were inoculated into culture medium and duration of survival of *T. foetus*

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was determined entirely by the results of the examination of these cultures.

Vaginal samples collected from a heifer during a period from seventeen to twenty-four days after she was bred to an infected bull were used as the source of trichomonads in another series of trials. Ten cubic centimeters of sterile 0.85 per cent saline were injected into the vagina and the fluid was collected with a pipette and rubber bulb as described by Andrews and Miller.[5] Five vials were set up similar to those of the first series except vaginal fluid was used as the source of trichomonads instead of the trichomonad culture and vials 1 and 2 were prepared by dividing the undiluted fluid remaining from the vaginal samples after vials 3 to 5 were inoculated. Also, 1,000 units of penicillin and 1 mg. of streptomycin were added to vial 5 in each trial. Each sample was examined at 24-hour intervals or less until all trichomonads had apparently disappeared. No estimates of concentration of trichomonads were made and no cultures were inoculated. In this series, seven trials were conducted.

RESULTS

As shown in table 1, active specimens of *T. foetus* were demonstrated for an average of 5.9 days in egg yolk citrate fluid containing sulfanilamide, penicillin, and streptomycin, and maintained at 39 F. In this fluid, without the penicillin and streptomycin, trichomonads were demonstrated for an average of 5.0 days. The average estimated concentration of trichomonads at the daily examinations was 8.7 and 9.9 thousand per cubic centimeter, respectively. These results indicate that the presence of penicillin and streptomycin under these conditions had little apparent effect on duration of survival of *T. foetus*.

In saline solution at 39 F., the average duration of survival was 7.2 days, whereas, in the culture mediums, trichomonads were still demonstrable after 11.1 and 10.9 days at 39 F. and room temperature, respectively. The average concentrations of trichomonads in these vials were 18.5, 33.5, and 245.1 thousands per cubic centimeter, respectively. The markedly higher number of trichomonads in culture medium at room temperature indicates the sensitivity of *T. foetus* to temperature differences.

The results with the cultural method were not consistently different from those with the direct examination. In several cases, as in trials 4, 6, and 8, trichomonads were demonstrated in the egg yolk fluids for two to six days longer with the cultural method than with the direct examination, but the averages of all trials were similar for the two methods.

In the second series of trials (table 2), it was found that in general *T. foetus* survived in the egg yolk buffer fluids slightly longer than activity of sperm was maintained. The averages for the buffer mixture and the buffer mixture with antibiotics was 8.3 and 8.7 days, respectively, for survival of *T. foetus*, while the corresponding averages for duration of sperm activity were 7.7 and 7.3 days. The duration of survival of trichomonads in saline solution was slightly shorter (6.7 days), while the duration of survival in culture medium at room temperature and at 39 F. was distinctly longer (14.3 and 12.7 days, respectively) than in the egg yolk buffer mixtures. The results of the second series of trials, al-

TABLE 1—Duration of Survival of Trichomonas Foetus from Cultures in Egg Yolk Buffer Mixtures as Used for Dilution of Semen

Trial No.	No. of days *T. foetus* demonstrated by direct examination and average No. of *T. foetus* in thousands per cc. per positive examination										No. of days *T. foetus* demonstrated by culture	
	Cult. med. room temp.		Cult. med. 39 F.		Saline solution 39 F.		Buffer mixture 39 F.		Buffer mixture plus antibiotics 39 F.		Buffer mixture 39 F.	Buffer mixture plus antibiotics 39 F.
1	12*	39.1	12*	14.8	7	7.2	1	6.7	5	4.9	.....	.....
2	11*	18.9	11*	10.5	8	5.9	4	0.1	2	2.4	.....	.....
3	12*	87.9	12*	62.0	8	19.8	3	3.0	3	10.3	3	4
4	10*	14.5	9	6.2	5	8.9	1	1.1	0		3	3
5**†	15*	435.4	15*	60.1	8	24.2	11	52.0	15*	21.3	15	14
6	14*	422.9	14*	38.6	10	32.4	5	10.0	5	11.4	11	8
7**	9*	67.2	9*	17.3	7	13.6	3	2.9	0		3	3
8	9*	62.9	9*	21.1	5	11.9	1	1.1	1	3.3	3	3
9	9*	78.8	9*	32.5	9*	10.4	3	4.9	5	1.6	4	4
10**	7	161.9	11	38.5	7	16.2	11	6.5	12	4.7	4	8
11**	12*	455.1	12*	51.1	8	48.2	11	32.2	12*	44.5	11	11
12**	9*	1,177.6	9*	36.2	9*	26.9	9*	5.6	9*	8.2	2	5
13**	11*	261.0	11*	31.0	3	12.9	2	3.3	6	2.9	4	6
14**	13*	148.8	13*	50.0	7	20.0	6	9.6	7	6.1	7	7
Ave.	10.9	245.1	11.1	33.5	7.2	18.5	5.0	9.9	5.9	8.7	5.8	5.9

* Trichomonads still demonstrable on last examination; ** 1,000 units penicillin and 1 mg. streptomycin added to vial 5; † Inadvertently left at room temperature for 24 hours on third day.

though limited in number, indicate that the presence of semen in the egg yolk buffer mixtures did not have an inhibiting effect on the survival of *T. foetus*. The results with the cultural method of examination in both series indicate that *T. foetus* is able to retain its ability to multiply, and therefore, presumably, its ability to establish infection in susceptible females after storage for approximately five to nine days at 39 F. in egg yolk buffer mixtures containing sulfanilamide, penicillin, and streptomycin.

The results with the experiment involving *T. foetus* from vaginal samples (table 3) differed in some respects from those with *T. foetus* from cultures. The average duration of survival in the egg yolk fluid with antibiotics was 9.6 days, in that without antibiotics it was 9.9 days, while in saline solution it was only 1.6 days. Therefore, the duration of survival of *T. foetus* from vaginal samples was somewhat longer in the egg yolk fluids and was distinctly shorter in the saline solution than was found for *T. foetus* from cultures. It is likely that the difference in duration of survival in saline solution was influenced by the presence of diluted culture medium in the experiment with *T. foetus* from cultures. The presence of antibiotics in the egg yolk fluids apparently did not affect the duration of survival of *T. foetus* from vaginal samples; this agrees with the finding for *T. foetus* from cultures.

In the two vials in which undiluted portions of vaginal samples were kept at room temperature or at 39 F. the average duration of survival of trichomonads was 4.0 and 3.7 days, respectively. Therefore, trichomonads did not survive as long in these vials as in the egg yolk fluids.

The results of the series of trials with *T. foetus* from vaginal samples and those of the two series of trials with *T. foetus* from cultures agree in indicating that this organism is able to survive in the fluids used for diluting semen for periods of five to ten days, well beyond the usual time involved in storage and transportation of the semen. Therefore, transmission of trichomoniasis by artificial insemination involving semen diluted with these fluids appears to be possible.

Results of Insemination with Diluted Semen from an Infected Bull.—In August, 1951, a Holstein-Friesian bull was purchased at a herd dispersal sale by the Cache Valley Breeding Association for use in its artificial insemination unit. Semen was collected from the bull, diluted 1 to 15 with egg yolk citrate mixture containing 6 mg. of sulfanilamide, 1,000 units of penicillin and 1 mg. of streptomycin per cubic centimeter, then shipped to three inseminators in Cache Valley, who used it to inseminate 32 cows and heifers, 1 of which was virgin. Two days after this collection of semen the bull was examined by the pipette method for trichomoniasis and was found to be positive. The concentration of *T. foetus* in the preputial samples was relatively low in comparison to the usual finding in infected bulls.

Fourteen to 20 days after they were inseminated the 32 females were examined for trichomoniasis. Vaginal samples were obtained by the method described above and were then examined under the microscope. All were negative. Twenty of the inseminations resulted in pregnancies and in all of these cases normal calves were delivered at full term. This is a conception rate of 62.5 per cent as compared with an average rate of 66.5 for 18 bulls at the center for the same month. Of the remaining 12 cows, 11 were settled as a result of subsequent services. Records on the twelfth cow were incomplete.

TABLE 2—Duration of Survival of Trichomonas Foetus from Cultures in Egg Yolk Buffer Mixtures Containing Bull Semen

Trial No.	No. of days *T. foetus* demonstrated by direct examination and average No. of *T. foetus* in thousands per cc. per positive examination						No. of days active sperm demonstrated by direct examination		No. of days *T. foetus* demonstrated by culture	
	Cult. med. room temp.		Cult. med. 39 F.		Saline solution 39 F.		Buffer mixture 39 F.	Buffer mixture plus antibiotics 39 F.	Buffer mixture 39 F.	Buffer mixture plus antibiotics 39 F.
1	15*	134.2	14	4.6	8	2.8	4	4	7	8
2	16*	746.7	13	31.5	4	3.8	11	7	6	7
3	12*	167.9	11	13.1	8	6.3	8	8	12*	11
Ave.	14.3	349.6	12.7	16.4	6.7	4.3	7.7	7.3	8.3	8.7

* Trichomonads still demonstrable on last examination.

The evidence thus indicates that no cases of infection resulted in the 32 females inseminated with the diluted semen from the infected bull. The infected bull was later

TABLE 3—Duration of Survival of Trichomonas Foetus from Vaginal Fluid in Egg Yolk Buffer Mixtures as Used for Dilution of Semen

Trial No.	No. of days *T. foetus* demonstrated by direct examination				
	Vaginal fluid room temp.	Vaginal fluid 39 F.	Normal saline solution 39 F.	Buffer mixture 39 F.	Buffer mixture and antibiotics 39 F.
1	2	2	2	15	15
2	2	4*	3	7	13
3	3	3	1	10	5
4	11	11	2	13*	8
5	9	3	3	10	10
6	0	2	0	7	9
7	1	1	0	7	7
Ave.	4	3.7	1.6	9.9	9.6

* Trichomonads still demonstrable on last examination.

treated successfully by a method described elsewhere [9] and returned to service in the unit.

DISCUSSION

The results of the experiments with *T. foetus* from cultures and from vaginal samples agree with those of Joyner and Millar [5] in indicating that trichomonads are able to survive for several days at low temperatures in egg yolk buffer mixtures containing antibiotics. In the experiments of Joyner and Millar,[5] the trichomonads in culture medium were added to fresh semen prior to diluting 1 to 10 with egg yolk buffer mixture containing 1,000 units of streptomycin per cubic centimeter. The mixtures were stored at 4 to 8 C. and samples were removed periodically for examination by a culture method involving glucose broth on an inspissated serum slope. In three trials, trichomonads remained alive from two to five days. In the present work, trichomonads were found to survive somewhat longer; the different sources of *T. foetus*, the daily examinations in most of the trials with associated warming of the samples to room temperature, and the different methods of examination used as compared with the study of Joyner and Millar may have influenced the difference in results.

Vandeplassche and co-workers [3] compared the results of use of semen from an infected bull with that from 2 uninfected bulls over a period of nine months. The semen was diluted 1 to 25 to 1 to 80 with an egg yolk citrate mixture containing sulfanilamide and used for three to four days; 600 females were inseminated with semen from the infected bull and a similar number with semen from the uninfected bulls. The results with respect to conception rate were similar for the infected and uninfected bulls, but in the females in which semen from the infected bull was used there were a few more cases of early abortion (5 as compared with 2), and a little more irregular estrous cycles in those not settled at the first insemination. One case of pyometra was found which was considered to have originated from the use of semen from the infected bull. The authors consider that the dilution of the semen was a factor of great importance in reducing the rate of transmission of trichomoniasis. With use of fresh undiluted semen from an infected bull, Garlick [1] observed 2 cases of transmission of trichomoniasis and Bartlett and co-workers [2] found that 6 of 9 previously uninfected females became infected.

Bartlett and co-workers [6] stated that a half-dozen instances had come to their attention which left no reasonable doubt that transmission by artificial insemination had occurred. On the basis of admittedly superficial observation, they found that apparently the rate of transmission may usually have been less than 1 per cent. They considered that the seriousness of trichomoniasis in relation to artificial insemination lies in the possibility of herd sires becoming infected from females infected by artificial insemination.

In the present study, the finding that no cases of infection resulted from the use of diluted semen from an infected bull on 32 females agrees with the results of Vandeplassche and with those of Bartlett in indicating that the rate of transmission of trichomoniasis involving diluted semen is low. The finding that *T. foetus* is able to survive in the mediums used in diluting semen indicates that, as Vandeplassche and coworkers suggested, the dilution of the semen is probably of great importance in reducing the rate of transmission from that characteristic of natural breeding or artificial insemination with fresh undiluted semen.

The indication that there is only a low rate of transmission of trichomoniasis by

artificial insemination involving the use of diluted semen from infected bulls does not imply that trichomoniasis is of no concern in artificial insemination. On the contrary, the fact that such transmission can occur justifies careful examination of bulls used for artificial insemination, and treatment of any infected with trichomoniasis.

SUMMARY

In 14 trials with *Trichomonas foetus* from cultures, the average duration of survival in egg yolk buffer mixtures with sulfanilamide, penicillin, and streptomycin at 39 F. was 5.9 days. In three trials under similar conditions, except that semen was present and examination for trichomonads was made by the cultural method, the average duration of survival of *T. foetus* was 8.7 days, while the average duration of sperm activity was 7.3 days. In seven trials with *T. foetus* from vaginal samples, the average duration of survival was 9.6 days. There was little difference in the duration of survival of trichomonads in the buffer mixtures which contained streptomycin, penicillin, and sulfanilamide and those which contained only sulfanilamide. Trichomonads survived for longer periods in culture medium than in the buffer mixtures, for about the same periods in saline solution when cultures were used as the source of trichomonads, but distinctly shorter periods in saline solution or in undiluted vaginal fluid when trichomonads came from vaginal samples.

The use of semen from an infected bull, diluted 1 to 15 in egg yolk buffer mixture containing sulfanilamide, streptomycin, and penicillin resulted in no cases of infection in 32 females, and the rate of conception was similar to that for uninfected bulls.

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A Study on the Relationship of Vitamin A to the Development of Hyperkeratosis (X Disease) in Calves

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REDUCTION of vitamin-A concentrations in blood plasma of cattle fed substances which produced hyperkeratosis was first reported by Hansel *et al.*[2] Liver storage of vitamin A was also low. These authors reported that vitamin A fed in large amounts was absorbed, and vitamin-A values in the liver and plasma increased during the period of vitamin-A administration. Since large doses of carotene increased carotene concentrations in the plasma but did not increase the vitamin-A values, they suggested that the hyperkeratosis factor may exert an antivitamin-A effect by interfering with the conversion of carotene to vitamin A. They did not report the effects of vitamin A on the course of the disease.

Webster *et al.*[7] discussed the effects of supplemental vitamin A in cattle which had typical symptoms and lesions of hyperkeratosis. It was concluded that vitamin-A therapy apparently was of definite value in delaying the appearance of, if not preventing, the disease in calves and alleviating the condition in older animals.

Field observations of cases of hyperkeratosis or x disease in cattle focused our attention upon the similarity of the syndrome with the symptoms of avitaminosis A in young cattle. These observations led to a survey study of the disease as it occurred in Wisconsin in the late winter of 1951-1952, and to controlled investigations of experimentally produced hyperkeratosis in young cattle.

EXPERIMENTAL DATA AND RESULTS

Field Studies on Hyperkeratosis and Vitamin A.—This survey was made in March, 1952, for the purpose of studying the nutritional status of individual animals in herds affected with the hyperkeratosis syndrome. Blood components which were studied were carotene, vitamin A, vitamin C, niacin, vitamin B_6, vitamin B_{12}, and the minerals, sodium and potassium. Animals with signs of hyperkeratosis from 15 herds, as well as a group from three unaffected control herds, were included in the study. Of the blood constituents studied, the only one significantly altered in hyperkeratosis was the vitamin A in the blood plasma. Although some of the other blood components studied were low in certain individual animals, the values obtained from the entire group overlapped and were inconsistent. It was of interest that the calves with evidence of hyperkeratosis had vitamin-C concentrations in the plasma slightly higher than the control animals. The mean concentration of vitamin C for 54 affected animals was 0.54 ± 0.20 mg. vitamin C/100 ml. of plasma compared to a mean of 0.42 ± 0.19 mg./100 ml. of plasma for 12 unaffected controls. The difference encountered approaches statistical significance as analyzed by the "t" test, the probability that the difference is due to chance lying between 0.10 and 0.05. This supports the results obtained by other workers.[4,5]

The summary of data on the vitamin-A concentrations in the plasma found in calves showing evidence of hyperkeratosis and in normal control calves is presented in table 1. About 70 per cent of the calves which showed varying degrees of symptoms related to hyperkeratosis had vitamin-A concentrations in the blood plasma of less than 10μg./100 ml. and were definitely suboptimal in vitamin A. Only 1 of 18 control calves had a similar suboptimal level of vitamin A. Many of the calves considered to have hyperkeratosis were being fed varying amounts of vitamin A under directions of the local veterinarians. This fact may have been responsible for some of the normal vitamin-A values encountered in the affected group.

Field tests were made to determine if supplementation of vitamin A in rather large amounts would effectively increase vitamin-A values in the blood plasma of calves with hyperkeratosis, and to ascertain the effect of such treatment on the course of the disease. It was found that supplementation with a total of 600,000 to 750,000 units of vitamin A over a nine- to twelve-day period usually resulted in a significant increase in vitamin A in the

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Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by the McMillen Feed Mills, Fort Wayne, Ind.

The authors acknowledge the help of the Wisconsin State Division of Livestock Sanitation under the direction of Dr. H. J. O'Connell, of Dr. S. H. McNutt of the Department of Veterinary Science, and of R. F. Miller of the Department of Biochemistry, and thank them for their assistance in certain phases of these investigations.

TABLE 1—Summary Data of Vitamin-A Concentrations in Blood Plasma of Young Cattle in Field Cases of X Disease (Hyperkeratosis)

Status of farm	No. farms	No. animals	Vitamin-A values in blood plasma (percentage of animals per class)		
			less than 10 μg./100 ml.	10-15 μg./100 ml.	more than 15 μg./100 ml.
X disease	15	63	68	22	10
No x disease	3	18	6	61	33

blood plasma to within the normal range. However, in some calves no change in plasma vitamin-A concentration occurred. Data on the effect of treating a typical field case of hyperkeratosis with vitamin A are presented in table 2.

Vitamin-A supplementation appeared to decrease diarrhea, to greatly decrease coughing, and to improve appetite and general condition, but it did not noticeably alter the lacrimation, mouth lesions, or skin condition over this short period of time. The effects of short-time vitamin-A therapy on vitamin-A concentrations in the plasma and on the condition of the calves was usually short-lived. The results suggested that temporary, partial alleviation of the severe cases of hyperkeratosis syndrome occurred on short-time vitamin-A therapy and only if the vitamin-A concentrations in the plasma were raised to within the normal range during the period of treatment.

Controlled Experiments on Hyperkeratosis and Vitamin A.—For these experiments, commercial pelleted calf feeds were obtained from eight different farms where hyperkeratosis had been encountered. These feeds were used to produce hyperkeratosis and will be referred to as x pellets. In a preliminary experiment, 6 calves of 1 to 2 weeks of age, which were fed x pellets and a limited supply of milk, died after fifteen to eighty days. They all had extremely low vitamin-A concentrations in the blood plasma whether or not additional vitamin A was given. Lacrimation and typical mouth lesions were observed in some of the calves, but death was always from terminal pneumonia. However, in this experiment, 2 of 3 control calves fed nontoxic pellets used in the university dairy herd also died after seventeen and thirty days with respiratory infection and low vitamin-A values in the plasma. Because of the prevalence of respiratory infections which resulted in death in the control calves, and because of insufficient clinical evidence of hyperkeratosis, no interpretation of the results was possible. However, on many farms where hyperkeratosis was encountered, calves were found which did not manifest the typical hyperkeratosis syndrome but had symptoms similar to those seen in the young experimental animals used in this study. It appears that young calves which consume a very limited amount of a toxic hyperkeratosis-producing feed develop vitamin-A deficiency, become susceptible to secondary infection, and die with a terminal pneumonia.

A second experiment was set up in June, 1952, under more desirable environmental conditions, using older and more vigorous calves. Nine healthy, Holstein-Friesian bull calves ranging in age from 2 weeks to 2 months were divided as equally as possible into three lots of 3 calves each. All calves received milk for six weeks (12 to 13 lb./day), a fair grade of mixed grass and alfalfa hay, and a grain mix. In lot 1 (control) the grain mix was composed of one-third corn, one-third oats, and one-third control, nontoxic pellets used in the university dairy herd. In lots 2 and 3, the x pellets replaced the nontoxic pellets. Calves in lot 3 each received orally by capsule 25,000 I.U. of vitamin A per day. Thus, the three experimental groups were: lot 1, control; lot 2, x pellets; and lot 3, x pellets plus vitamin A. The calves were housed in a clean shed, each pen having an outside run. Lots 2 and 3 were placed in one pen while lot 1 occupied an adjacent pen. The experimental rations were offered after a twelve-day stabilization period, and milk feeding was discontinued one month after the calves were put on the experimental rations.

The calves were weighed, and the carotene and vitamin-A concentrations in the blood plasma were determined at weekly intervals. The animals were observed daily and their status critically evaluated once each week.

TABLE 2—Data Showing the Effect of the Administration of Vitamin A* in a Field Case of X Disease

Treatment	Vitamin-A concentrations in plasma (μg./100 ml.)**		
	Before treatment	At end of nine day treatment	Seven days after treatment ended
Placebo	5.7	8.0	8.3
Vitamin A	3.2	11.4	8.4

* 750,000 units of vitamin A were given over a period of nine days. ** Averages of 5 calves on each treatment.

One calf in lot 2 (x pellets) died of acute lindane poisoning seven days after the calves were started on the experimental rations, and attempts to control flies thereafter were discontinued. Other calves in the group apparently were not affected by the lindane.

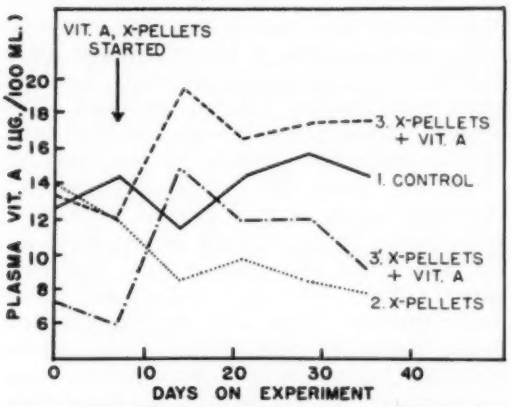

Fig. 1—Effect of feeding x pellets during the milk-feeding period upon vitamin-A concentrations in the blood plasma of calves.

Fluctuations in carotene concentrations in the plasma were variable and inconclusive but seemed to be dependent upon hay consumption. These data are therefore not presented. Figures 1 and 3 present the plasma vitamin-A data for the three lots of calves and figures 2 and 4 present the body weight data. The experiment has been divided into the milk-feeding period and the post milk-feeding period to facilitate interpretation of the results. Lot 3 (x pellets plus vitamin A) has been subdivided to include lot 3 and lot 3', since the 1 calf (3') behaved so differently from the other calves. The significance of this differential response is considered later.

It should be pointed out that while the calves were consuming milk, the intake of grain mix in all lots was small; the calves consumed on the average about 0.2 lb. of pellets per calf per day. Thus, the trends in vitamin-A levels in the plasma during this period are the result of ingestion of small amounts of a hyperkeratosis-producing feed. From figure 1 it may be seen that during the milk-feeding period, control calves (lot 1) maintained a vitamin-A concentration in the plasma of about 14 μg./100 ml., while the concentration of vitamin A in the plasma of calves receiving the x pellets without vitamin A dropped to about 8 μg./100 ml. Chemical analyses of the x pellets and the control pellets showed them to contain about the same amount of vitamin A (3,000 and 2,600 I.U. of vitamin A/lb., respectively). Since the feed intake was essentially the same in the two lots during this period, the vitamin-A intake was about the same. In lots 3 and 3', vitamin-A values in the plasma climbed sharply upon supplementation of 25,000 I.U. of vitamin A per day. The 2 calves receiving x pellets plus vitamin A (lot 3) which survived the experiment maintained a vitamin-A concentration in the plasma above that of control calves during the milk-feeding period (about 17 μg./100 ml.), while the vitamin-A concentration in the plasma of the calf which later died (lot 3') dropped to 9 μg./100 ml. after an initial rise. These data show that with an intake of a limited amount of a hyperkeratosis-producing feed, a calf fed this amount of supplemental vitamin A was capable of maintaining a normal vitamin-A concentration in the plasma. In this experiment, the grain mix was offered free choice to the groups of calves; thus, no estimate of the pellet consumption of each individual was obtained. It appeared during this experiment that the calf designated lot 3' consumed more grain than the other calves, but we have no proof on this point. This factor probably explains the difference in behavior of the lot 3' animal.

Figure 2 shows that during the milk-

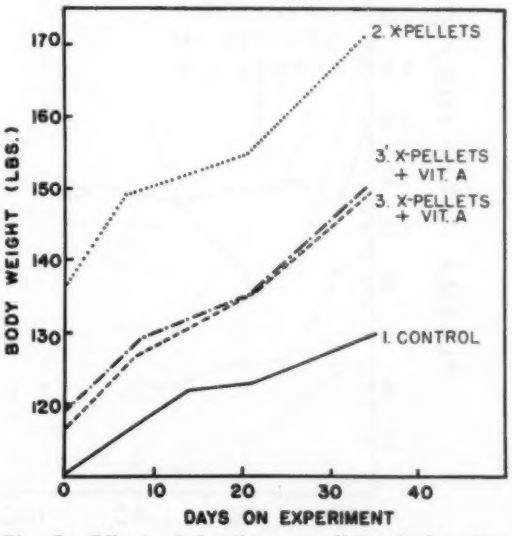

Fig. 2—Effect of feeding x pellets during the milk-feeding period upon body weight gains of calves.

feeding period the calves fed x pellets, with or without vitamin A, gained in body weight as well or better than control calves fed nontoxic pellets. No symptoms of hyperkeratosis were observed in any of the calves by the end of this period. The ability of the calves fed toxic pellets to maintain good weight gains was probably due not directly to the consumption of milk but to the limitation in intake of the toxic principle carried by the pellets.

With the discontinuance of milk feeding, pellet consumption was increased to about 1 lb. of pellets per calf per day, but this dropped sharply in lots 2, 3, and 3′ as appetite decreased. During the entire experiment, the calves of lots 2, 3, and 3′ consumed an average total of 18 lb. of pellets per calf. During the post milk-feeding period (fig. 3 and 4) the vitamin A in the plasma of control calves dropped to about 11 μg./100 ml. but was maintained at this level throughout the remainder of the experiment. These control calves meanwhile made good weight gains. Calves in lot 2 (x pellets without vitamin A) showed rapidly decreasing plasma vitamin-A concentrations to critically low amounts (less than 2 μg./100 ml.). They immediately began to lose weight and died, showing typical lesions of hyperkeratosis after seventy-three and seventy-six days on experiment or thirty-eight and forty-one days after milk feeding was stopped. Although the lot 3′ calf received vitamin-A supplementation, its plasma level of vitamin A continued to decline to about 3 μg./100 ml. Body weight was maintained, but the calf died with typical lesions of hyperkeratosis after 101 days on experiment or sixty-six days after milk feeding was discontinued. The other 2 calves which received 25,000 units of supplemental vitamin A per day, or about five times the normal requirement, showed a sharp drop of plasma vitamin A from about 18 μg./100 ml. to below 8 μg./100 ml. when more grain mix was consumed. They also just maintained their body weights. These data demonstrate the potent antivitamin-A effects of the hyperkeratosis factor. When the x pellets were replaced by control pellets, an immediate increase in vitamin A in the plasma followed, reflecting again a direct antagonism of the x factor on vitamin A. These calves recovered and about two weeks after the x pellets were removed from the ration they were making weight gains comparable to those made by control animals.

Symptoms and lesions of hyperkeratosis

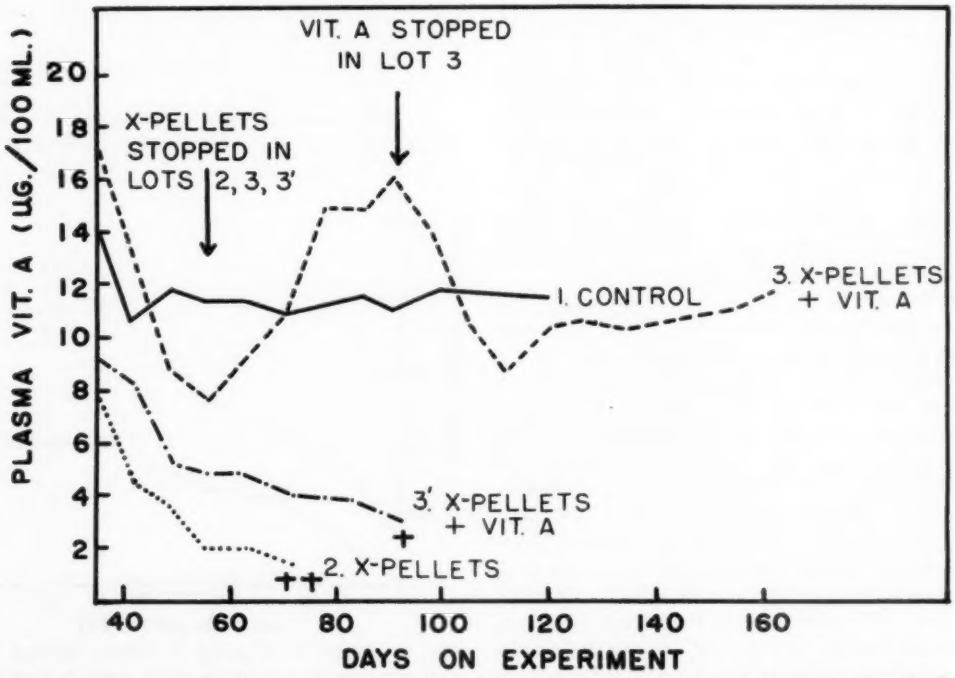

Fig. 3—Effect of feeding x pellets during the post milk-feeding period upon vitamin-A concentrations in the blood plasma of calves (+ indicates death).

began to appear in all the calves in lots 2, 3, and 3′ after about six weeks on experiment, or one week after milk feeding was discontinued. The following symptoms were among those observed. Those symptoms and lesions observed in all the calves fed x pellets are marked with an asterisk (*).

*1) There were depressed vitamin-A values in the blood plasma;
*2) profuse lacrimation evidenced by tear tracks on the side of the face;
*3) loss of body weight, depression, and listlessness;
*4) a variety of mouth lesions including reddened areas, papillomas, proliferations, and ulcerations;
*5) coughing;
6) diarrhea;
7) drying, thickening, and wrinkling of the skin along the neck and shoulders;
8) on autopsy, consolidation of the lungs, ulcerative lesions in the gastrointestinal tract, distention of the gall bladder, slight degeneration of the liver, and congestion of the kidneys.

The symptoms in lot 3 were definitely less severe than in lot 2; however, the vitamin-A supplementation did not prevent the development of a typical hyperkeratosis syndrome.

In figure 3, it may be seen that when supplementation of vitamin A ceased in lot 3 five weeks after the x pellets had been removed from the ration, a rather sharp drop in plasma vitamin A occurred to a point below that of control calves. From the weight curves, it is apparent that these calves were gaining as well as the control animals. It appears that the toxic factor continued to exert a slight depressing effect on the vitamin A in the blood plasma more than five weeks after cessation of feeding.

DISCUSSION

The severe antagonism of the factor or factors responsible for hyperkeratosis to vitamin A in the calf has been demonstrated. The question arises whether or not the symptoms and lesions of hyperkeratosis are a direct result of the lowered vitamin A. It is significant that we have observed most of the symptoms and lesions of hyperkeratosis in experimentally produced vitamin-A deficiency. The papilloma-like oral lesions and the extreme thickening and keratization of the skin have not been observed in experimental vitamin-A deficiency, although similar, but less pronounced, changes sometimes occur. It is our belief that the antagonism of the toxic factor on vitamin-A metabolism is a primary factor in producing the hyperkeratosis syndrome. Vitamin A was always lowered in untreated hyperkeratosis. The helpfulness of vitamin-A therapy depends on whether or not the vitamin-A values in the plasma can be raised to essentially normal values. From our experiments, it was apparent that supplemental vitamin A was beneficial in hyperkeratosis. It increased survival, but it did not effect a cure.

How the toxic hyperkeratosis factor causes a depletion of vitamin A is not known. The Cornell [2] workers suggested that interference of the conversion of carotene to vitamin A may be important in lowering vitamin-A concentrations in the plasma in hyperkeratosis. Our data on carotene levels, and the fact that vitamin A in the plasma was depressed when vitamin A itself was administered during the development of hyperkeratosis, suggests that this is not its primary mode of action. There are several possibilities which may explain the depressed vitamin A. Among these are (1) inhibited absorption, (2)

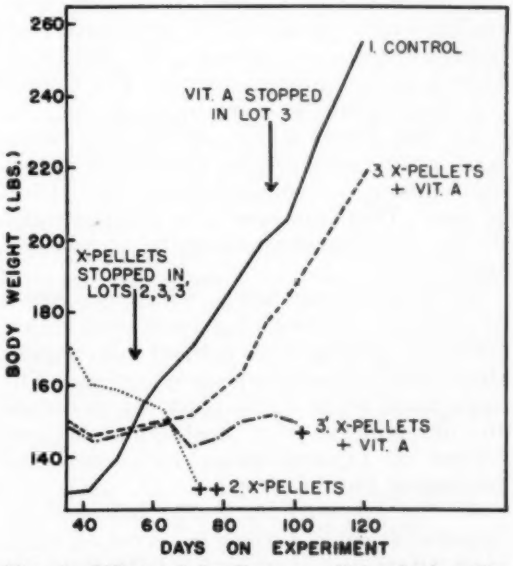

Fig. 4—Effect of feeding x pellets during the post milk-feeding period upon body weight of calves (+ indicates death).

greatly increased excretion, (3) chemical destruction or inactivation of the vitamin-A molecule, (4) an "antivitamin-like" action, or (5) a greatly increased requirement for vitamin A.

The "antivitamin A-like" action of the hyperkeratosis factor, which may occur in cattle, appears not to operate in the chicken, since administration of an ether extract of the x pellets to chicks in amounts 50 times that given to a calf (body weight basis) had no depressing effect of vitamin A in the liver. The calf meanwhile showed a severe depression of vitamin A in the blood plasma.[3] Depressed vitamin A in the liver occurs in calves fed hyperkeratosis-producing feeds.[2,3] Mice and rats were apparently unaffected by our hyperkeratosis-producing feeds. We have been unable to show a depression of vitamin A in homogenized fresh cow liver or in corn oil after as much as twenty hours of incubation *in vitro* at 37 C., with either ether extracts of hyperkeratosis-producing pellets, or with highly chlorinated naphthalenes,[3] such as those reported as being causative agents of hyperkeratosis.[1,5]

Further study is needed on the hyperkeratosis-vitamin A relationship. Such studies may give some insight into the actual function of vitamin A in the animal body.

SUMMARY

Data from field survey studies and controlled experiments on the relationship of hyperkeratosis (x disease) to vitamin A have been obtained in calves. The following observations seem to be important:

1) The factor which caused hyperkeratosis produced a severe depressing action on vitamin-A concentrations in blood plasma. There appears to be a definite and direct hyperkeratosis-vitamin A relationship.

2) Very young calves which consumed hyperkeratosis-producing feeds died with a terminal pneumonia without developing the typical hyperkeratosis syndrome. The same feeds when fed to older calves caused the development of typical symptoms and lesions of hyperkeratosis with continued feeding of the toxic feed.

3) Partial, temporary alleviation of the hyperkeratosis syndrome occurred on short time vitamin-A therapy (600,000 to 750,000 I.U. over a nine- to twelve-day period). If the vitamin-A concentrations in the blood plasma returned to normal during the vitamin-A therapy period and were maintained following treatment, a favorable prognosis was in prospect. On the other hand, if the low vitamin-A concentrations in the blood plasma failed to respond to vitamin-A administration during treatment or were unsuccessfully maintained after the therapy period, the hyperkeratosis proceeded to a slow but fatal termination.

4) Normal vitamin-A concentrations in the blood plasma were maintained in calves consuming limited amounts of hyperkeratosis-producing feed by supplementation with 25,000 I.U. of vitamin A per day (five times the normal requirement). However, when the intake of hyperkeratosis-producing feed was increased, 25,000 I.U. of vitamin A per day did not maintain vitamin-A concentrations in the plasma.

5) Supplementation with vitamin A (25,000 I.U./day) throughout the feeding of hyperkeratosis-producing feeds resulted in greater livability or prolongation of life but did not prevent the disease syndrome or its fatal termination in severely poisoned animals. If the disease were diagnosed early before gross symptoms of hyperkeratosis were observed, or if large amounts of the toxic material had not been consumed, and the toxic feed was removed, supplementation with vitamin A was beneficial.

6) The determination of vitamin-A concentrations in the plasma is valuable in the diagnosis of hyperkeratosis, and in following the progress of the disease in its development and regression.

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Production of Hyperkeratosis in Calves with a Topically Applied Oil-Based Insecticide Carrier

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BOVINE hyperkeratosis or x disease was first extensively described as a disease entity by Olafson.[7] Since that time, the disease has been recognized as a toxicity rather than an infectious disease or a simple nutritional deficiency. Among the numerous agents which have been reported as being responsible for outbreaks of bovine hyperkeratosis are the following: (1) certain processed feeds,[5, 8, 9, 10] (2) specific farm grown feeds,[6, 12] (3) a particular lot of wood preservative,[13] and (4) certain lubricants.[1] Bell[2] demonstrated that a highly chlorinated naphthalene, a chemical additive to the lubricant, was responsible for the symptoms and lesions of bovine hyperkeratosis. Sikes and Bridges[11] also reported the production of hyperkeratosis with pentachloronaphthalene. Hansel *et al.*[4] have isolated a hyperkeratosis-active compound from the wood preservative mentioned above and have identified it as trichloronaphthalene along with some more highly chlorinated naphthalenes as well.

In the summer of 1952, several cases of hyperkeratosis were observed which could in no logical manner be related to contaminated feedstuffs and, in numerous cases, oil-based insecticides had been heavily applied to the affected calves. Experiments were organized to test some of the more commonly used types of contact, oil-based insecticides for possible hyperkeratosis-producing activity on calves. It was apparent from these experiments that a commonly used, oil-based insecticide vehicle may produce a hyperkeratosis-like disease in calves.

EXPERIMENTAL

Twelve Holstein-Friesian bull calves 2 to 6 months old were divided into six experimental lots. They were housed in separate but adjacent pens and were fed a practical ration composed of fair quality grass and alfalfa hay, and a grain mix composed of corn, oats, and a commercial calf feed mix. After an initial pretreatment period of three weeks, the various lots were treated as follows: lot 1—control; lot 2—sprayed with 0.05 per cent lindane in mineral seal oil†; lot 3—sprayed with 0.5 per cent methoxychlor in mineral seal oil†; lot 4—sprayed with 3.2 per cent lethane and 2.3 per cent thanite (thiocyanate compounds) in mineral seal oil†; lot 5—x disease controls receiving orally 0.1 Gm. of highly chlorinated naphthalene in corn oil per day for six days; lot 6—sprayed with mineral seal oil only.†

The oil-based insecticides or oil were applied with hand sprayers onto the calves and on the walls of the pens. The amounts applied and the frequency of application are presented in table 1. It is estimated that about two thirds of the total amount of spray applied actually contacted the calves. Vitamin A concentrations in the blood plasma were determined at weekly intervals. The calves were observed daily and were critically evaluated once each week with respect to general condition and specific symptoms of hyperkeratosis. At the time of slaughter or death of the animals, the livers were analyzed for vitamin A content.

RESULTS AND DISCUSSION

The calves in lot 5 developed some of the symptoms of hyperkeratosis but did not show skin lesions. All calves in lots 2, 3, 4, and 6 developed hyperkeratosis. Wrinkling of the skin over the neck and shoulders was evident within one week after the initiation of treatment. The skin lesions developed eventually into the extreme thickening, folding, hardening, and cracking of the skin which is characteristic of bovine hyperkeratosis. Figures 1, 2, and 3 show these conditions in 2 of the calves which were sprayed with the oil-based insecticides and are more or less characteristic of all the calves in lots 2, 3, 4, and 6. The poor condition shown in figure 3 was exhibited especially by the calves which were the youngest at the time of treatment. The older calves maintained their condition

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Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by the McMillen Feed Mills, Fort Wayne, Ind.

† This oil has the following specifications:

Distillation		Viscosity (Saybolt)	
Initial	509	at 100 F.	44
End point	664	Unsulphonatable	
Gravity, API	36.8	residue	90+
Flash, degrees F. (Cleveland open cup)	265		

Fig. 1—Calf 78 of lot 2 (lindane in mineral seal oil) shows the extreme hyperkeratosis on the neck.

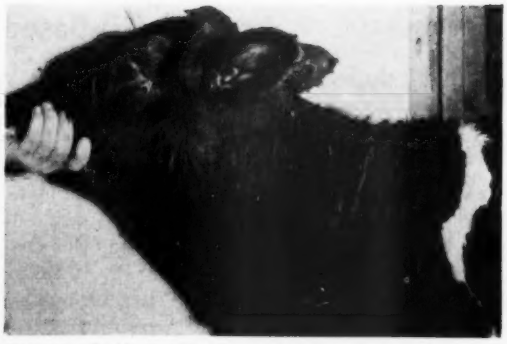

Fig. 2—Calf 17 of lot 4 (thiocyanates in mineral seal oil) shows the hyperkeratosis and moderate lacrimation.

Fig. 3—Calf 17 of lot 4 shows the poor condition exhibited by many of the calves treated with the oil-based insecticides or oil alone.

fairly well and exhibited the hyperkeratosis to a lesser degree. Neither of the control animals (lot 1) showed skin changes. Although most of the calves which developed the hyperkeratosis lacked condition, feed consumption in general was not severely affected. Some lacrimation was observed, and proliferative, ulcerative, and abrasive lesions developed on the oral mucosa and tongue.

The depressing action of orally administered hyperkeratosis-producing agents on the vitamin A concentrations in the blood plasma has been well established.[3-5] The blood plasma vitamin A data for the calves of the six experimental lots are shown in table 1. Four of the calves (marked with an asterisk [*] in table 1) were given 100,000 I.U. of vitamin A for three days just after the first bleeding to raise their subnormal vitamin A concentrations in the plasma. It should be mentioned that calf 10 of lot 1 (control) had been used previously in testing an extract of a hyperkeratosis-producing feed and had not completely recovered. The vitamin A supplementation in this case increased the vitamin A in the plasma only temporarily. Thus, this animal can not be considered as a normal control in respect to its plasma vitamin A trends. None of the calves treated with oil or oil-based insecticides had been used in previous experiments. A clear-cut, consistent drop in plasma vitamin A occurred within a week following the first application of the oil in all lots sprayed with the oil-based insecticides or the oil alone. Feed consumption meanwhile remained good. The vitamin A depression in the plasma, however, was not as sharp as in the case of orally administered, hyperkeratosis-producing agents (illustrated by lot 5 and our previous work). In general, the vitamin A values in the plasma did not reach a critically low point and increased as the dose and frequency of application of oil (or oil-based insecticide) was decreased. Vitamin A concentrations in the liver, although variable, indicate that vitamin A storage in the body had not been depleted in these lots at the time of slaughter. One calf (No. 14 of lot 3) was killed in a moribund condition during the fifth week of insecticide application and had an extremely low blood plasma vitamin A level. The condition of the calf was diagnosed as hyperkeratosis by the Wisconsin State Disease Control Laboratories. No gross lesions were observed in the gastrointestinal tract, gall bladder, or kidneys; however, there was degeneration of the liver and extreme consolidation of the lungs.

The mineral seal oil used in this experiment is a commonly used insecticide vehicle. It is reported to be a petroleum fraction with no additives. In our study, the addition of active insect toxicants to the oil did not enhance the toxicity.

The question arises whether our dosage of oil was excessive. Assuming that two thirds of the total spray used was applied to the calves, our heaviest dosage (first three weeks) corresponds to about 40 ml.

TABLE 1—Data on Vitamin A in the Blood Plasma and Liver

Week	No. times** sprayed during week following bleeding	Oil applied per calf + pen during week (ave. ml.)**	Blood plasma vitamin A conc. (μg./100 ml.)											
			1 Control		2 Lindane in oil		3 Methoxychlor in oil		4 Thiocyanate in oil		5 Chlorinated naphthalene		6 Oil only	
			8	10	78	79	14	15	16	17	7	516	18	20
1	0	.....	10.5	5.2*	5.5*	13.7	5.0*	10.9	14.9	6.3*	9.1	11.4	11.3	15.3
2	0	.....	11.6	14.4	11.6	17.1	12.2	11.8	.....	14.9	8.7	9.5	11.9	12.6
3	0	.....	13.3	12.5	12.3	17.9	11.9	8.2	17.0	10.0	10.3	12.2	11.6	.....
4	2	139	11.9	5.0	12.5	18.2	14.0	8.0	15.3	13.4	11.2	13.4	9.8	13.4
5	3	193	11.8	8.0	5.4	12.6	7.0	6.8	11.5	10.4	3.3	3.8	7.6	10.7
6	3	168	12.1	7.0	3.2	11.3	4.8	4.9	10.2	7.5	1.0	1.6	8.3	9.7
7	2	141	14.0	7.0	5.0	11.9	2.4	6.8	10.9	6.9	0.9	1.2	5.1	10.4
8	1	64	11.2	6.4	7.9	11.0	1.4	5.5	12.0	7.2	2.0	.....	3.8	9.6
9	1	82	12.3	7.6	12.9	14.4	.....	8.3	12.6	10.2	2.2	.....	5.3	10.2
10	0	.....	13.4	3.4	14.2	13.6	.....	7.0	14.9	11.0	2.3	.....	4.5	13.4
11	0	.....	14.5	6.5	16.3	18.8	.....	13.0	17.0	11.5	3.3	.....	10.0	11.0
Liver vitamin A at death or slaughter†			5.0	2.5	22.4	6.7	3.7	6.0	8.8	10.5	0.5	.....	7.6	2.4

* Given 100,000 I.U. vitamin A for three days; ** refers only to lots 2, 3, 4, and 6; † μg./Gm. fresh weight.

per calf per application applied three times a week. Computed on the average daily basis this would be about 17 ml. per calf per day or approximately ¼ oz. per 100 lb. of body weight per day. This is double the recommended safe level of application as indicated by the directions of the manufacturer.

The hyperkeratosis produced by the mineral seal oil differed from that produced by our hyperkeratosis-producing feed [5] or the highly chlorinated naphthalene in this experiment, in that the vitamin A drop in the blood plasma was less, the appetite was not generally affected, and the hyperkeratosis of the skin appeared quickly as the first detectable sign on the administration of the oil. The chlorinated naphthalene-fed calves in this experiment showed a rapid and severe depression of plasma vitamin A, profound loss of appetite, profuse lacrimation, and mouth lesions. One calf which died (516) had hemorrhagic enteritis, a granular appearance of the inner gall bladder wall, and severe lung consolidation. No skin changes were detectable in either of these calves under these conditions. The differences in the characteristics of the hyperkeratosis syndrome may be due to the difference in means of administration of the toxic factor (oral vs. external) or may possibly represent a difference in the type of chemical toxicant itself. Hansel and coworkers [4] have described this same variation in the characteristics of the hyperkeratosis syndrome by external exposure to the German wood preservative as opposed to its oral administration.

SUMMARY

Hyperkeratosis was produced in calves by topically administered oil-based insecticides. Mineral seal oil, a common insecticide carrier, produced the clinical skin condition of similar degree whether or not active insect toxicants (lindane, methoxychlor, thiocyanate compounds) were dispersed in the oil at recommended levels.

Blood plasma vitamin A concentrations were somewhat depressed upon application of the oil or oil-based insecticides but, in general, they did not reach the critically low levels observed when highly chlorinated naphthalene or toxic pelleted feeds were administered orally. Likewise, the condition produced in this experiment had less effect on the appetite and general well-being of calves than that produced with toxic feeds.

The importance of dosage and frequency of application of this petroleum fraction in producing hyperkeratosis in calves is being further investigated.

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Studies on Toxic Factors Produced by Rumen Microorganisms of Sheep

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RECENT WORK by Dougherty and Cello[1] indicates that toxic nongaseous factors are present in rumens of normal ruminants, as well as in cattle and sheep having atony of the rumen. Intravenous injection of the supernatant fluids of rumen contents into dogs quickened the respiration and markedly depressed the blood pressure. In sheep, similar injections decreased rumen motility. Although some reaction was present following injection of fluids from normal ruminants, the physiological changes were much more marked with fluids from cattle and sheep clinically affected with bloat.

The present study was undertaken to confirm the presence of such factors and to discover their immediate source. Previous microbiological studies on the rumen contents of sheep clinically affected with rumen disorders had shown a numerical change in certain types of bacteria. In view of such results, a possible microbiological source of the toxic factors was examined.

MATERIALS AND METHODS

Rumen Samples.—Ingesta samples were removed from both live and dead sheep. In all cases, sterile 1-liter flasks were used for collecting and transporting the samples. No special precautions were used during the period of transportation to the laboratory to prevent agitation or aëration. Doetsch, Robinson, and Shaw[2] have indicated that aëration of ingesta samples results in no appreciable change of bacterial populations for at least four hours. In the present study, the period from collection to the laboratory was never greater than thirty minutes.

In live subjects, a stomach tube and pump were used to obtain ingesta samples. About 500 Gm. of ingesta were obtained from each animal. Animals dead of rumen disorders were autopsied and ingesta samples removed directly. A flame-sterilized spatula was used to place the ingesta into flasks.

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Portion of a thesis presented to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy.

The author is now in the Department of Bacteriology and Parasitology, New York State University, Upstate Medical Center, Syracuse.

TABLE 1—Effects of Injections of Filtrates on the Blood Pressure of Experimental Sheep

Filtrate (No.)	Filtrate (type)	Anesthesia	Change (mm.Hg)	Length of effect (min. and sec.)
2-C2	pure	procaine	+40	6.0
1-c	pure	sod. pent.*	+10	6.0
3	mixed	sod. pent.	+12	5.24
3	ingesta	sod. pent.	+40	20.0
1-c	pure	procaine	+100	10.0
5	mixed	procaine	+25	5.10
5	ingesta	sod. pent.	+30	5.30
6	ingesta	sod. pent.	+20	4.15
5-c	pure	sod. pent.	+30	5.40
ave.			+34	7.36
6	mixed	sod. pent.	−30	10.30
4	mixed	procaine	−25	3.30
5-c	pure	procaine	−20	7.0
2-C1	pure	procaine	−20	5.0
ave.			−24	6.30

* sod. pent. = sodium pentabarbital.

A total of about 1,000 Gm. was taken from each subject. Samples from normal animals were similarly obtained at the abattoir. Only animals seen immediately after death were used in these studies.

Preparation of Cultures and Filtrates.—Both mixed and pure cultures were used. A fluid thiol (Difco) medium was inoculated with raw ingesta

TABLE 2—Effects of Injections of Filtrates on the Blood Pressure of Experimental Dogs

Filtrate (No.)	Filtrate (type)	Anesthesia	Change (mm.Hg)	Length of effect (min. and sec.)
1-c	pure	seconal	+100	10.5
6	ingesta	sod. pent.*	+ 40	3.0
6-g	pure	sod. pent.	+ 60	10.30
3-a	pure	sod. pent.	+ 50	4.10
352-6	pure	sod. pent.	+ 40	15.0
4-c	pure	sod. pent.	+ 40	10.0
6-a	pure	sod. pent.	+ 30	4.0
ave.			+ 51	8.6
5-f	pure	sod. pent.	− 60	4.0 died
4	mixed	seconal	−100	5.6
352	mixed	sod. pent.	− 60	3.5
4	mixed	seconal	−120	6.0 died
4-c	pure	sod. pent.	− 50	6.30
452-1	pure	seconal	− 40	6.20
752-8	pure	sod. pent.	− 40	6.40
453-3	pure	seconal	− 60	3.30
5-f	pure	seconal	− 70	12.30
752-7	pure	sod. pent.	−140	20.0
752-5	pure	seconal	−100	25.0 died
752-4	pure	sod. pent.	− 60	20.0
ave.			− 75	10.0

* sod. pent. = sodium pentabarbital.

Fig. 1—Thin branching gram-positive rods seen in fresh rumen samples. This type of organism produced a toxic factor that elevated blood pressure in experimental sheep and dogs. × 1,000.

(approximately 1 Gm. of ingesta per 100 ml. of medium). After ten to twelve days' incubation at 37 C. in stoppered 250-ml. flasks, the medium was filtered through Seitz or Berkefeld filters. The filtrate was stored at 5 C. in brown sleeve-stoppered bottles before use.

Fig. 2—Thick gram-positive, spore-forming rods seen in fresh rumen samples. This type of organism produced a toxic factor that caused a depression of blood pressure in experimental sheep and dogs. × 1,000.

Pure cultures were isolated by streaking agar plates with ingesta or mixed cultures. Previous studies showed the best mediums for isolation were albimi and thiol agars. Single colonies were then picked to thiol broth and maintained in stock on this medium. All plates were incubated in an anaërobic jar for ten to twelve days at 37 C. Filtrates were prepared from pure cultures.

Injection of Experimental Animals.—The *in vivo* effects of toxic factors in filtrates were measured by intravenous injection in dogs and sheep. Direct measurement of carotid blood pressure and indirect respiration recordings were used. A Phipps and Bird direct-writing ink kymograph was used. Mercury-type manometers with a sodium citrate-blood continuation were employed. Recordings were made at a speed of 4 in. per minute.

For respiration records, a chest-type pneumatic bellows was used. A diaphragm manometer and attached pen recorded changes in rate and depth of respiration.

Injections were made in the jugular vein on the same side as the cannulated carotid artery. Usually the left vessels were used. Either sodium pentabarbital or seconal was used for general anesthesia. Some sheep were injected under local procaine hydrochloride anesthesia.

RESULTS

The results of injections of filtrates of mixed and pure cultures into sheep are shown in table 1. Only cultures showing a blood pressure response were selected for tabulation. Approximately 50 pure cultures were discarded after negative trials.

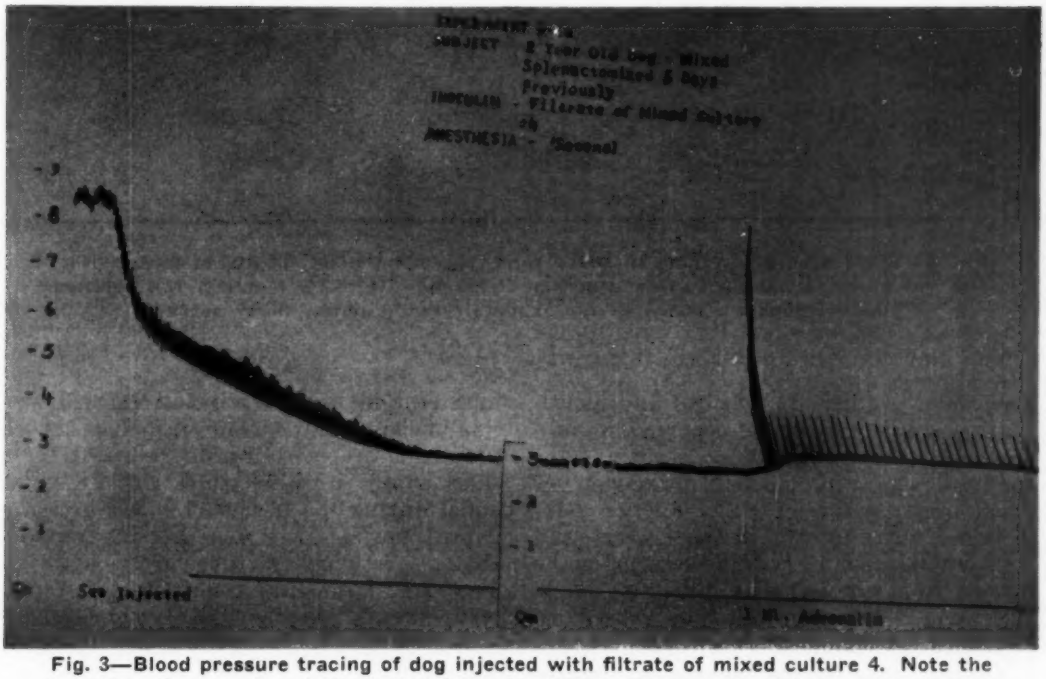

Fig. 3—Blood pressure tracing of dog injected with filtrate of mixed culture 4. Note the injection of adrenalin. Animal died following this temporary stimulation of blood pressure. Death time was approximately four minutes.

It was immediately obvious that two types of responses were involved. The blood pressure depressing action is similar to that described by Dougherty and Cello.[1] An additional factor was also present that caused a marked rise in blood pressure. The effects following injection in experimental dogs were similar (table 2).

Microscopic studies on the cultures producing positive changes were made. Pure cultures producing raises in blood pressure were thin, branching, gram-positive spore-forming rods (fig. 1). The organism producing the depressing action was, in all cases, a thick, short gram-positive spore-forming rod (fig. 2). Both species are

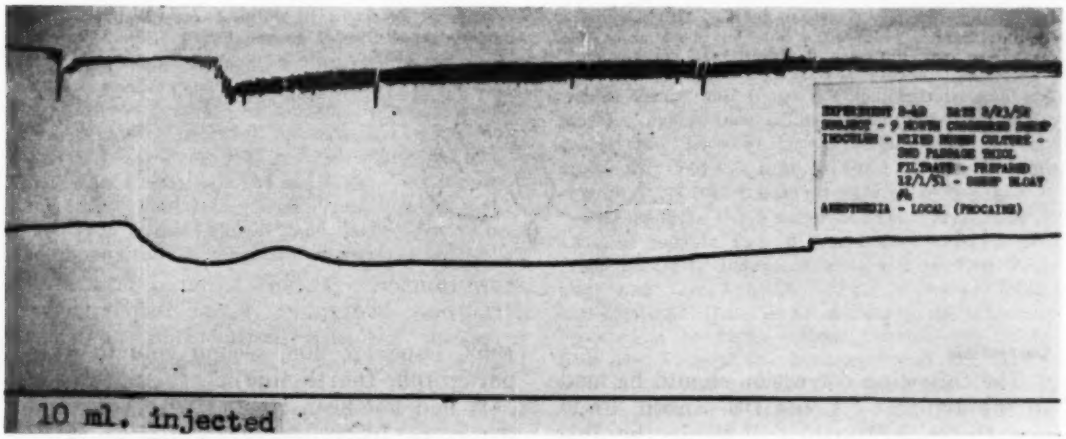

Fig. 4—Blood pressure (middle line) tracing of sheep following injection of filtrate of mixed culture 4. A low depression of 25 mm. Hg was obtained. Respiratory changes were marked only at the time of injection.

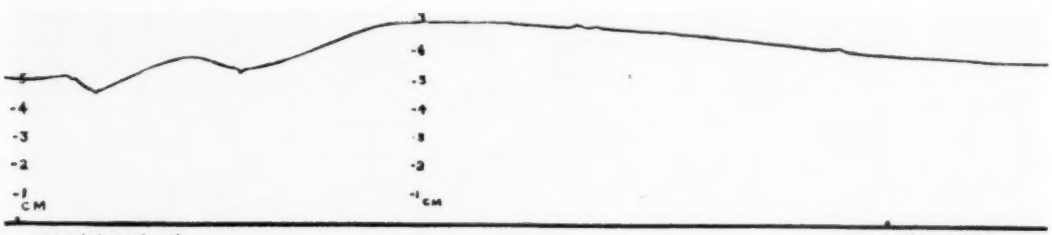

Fig. 5—Blood pressure tracing in sheep following injection of filtrate of pure culture 2-c. The pressure was raised from a normal of 100 mm. Hg to 140 mm. Hg. Normal pressure was again reached in approximately two minutes, thirty seconds.

anaërobic. Additional cultural and identification studies are to be reported in subsequent papers.

In all cases, an initial depression of blood pressure was obtained following injection of filtrates of both types, sheep and dogs reacting similarly. The reason for the initial drop was not determined. Control injections of medium filtrates caused no physiological changes.

Typical records are shown in figures 3 to 5.*

Respiratory changes were marked immediately following injection. They occurred simultaneously with the initial blood pressure change. In all cases, respiration was increased in both depth and rate. Injection of depressant filtrates into dogs caused a sudden deep inspiration followed by a short pause. Forced inspirations were also noted during the period of blood pressure depression. In all cases, respiratory changes returned to normal in approximately half the time required for circulatory changes to return to normal levels.

* In figure 3, a drop of 100 mm. Hg was noted following injection. In this animal, death occurred in approximately four minutes. As with most of the experimental dogs, the spleen was previously removed. Figure 4 shows the effect of the same filtrate on an experimental sheep. A depression of 25 mm. Hg was obtained. The blood pressure changes from most filtrates were less pronounced in sheep than dogs. Figure 5 demonstrates a typical change following the injection of a blood pressure stimulating filtrate. A rise of 40 mm. Hg was noticed. Similar filtrates produced a like, but more pronounced, change in blood pressure of experimental dogs (table 2).

SUMMARY

Studies on toxic factors produced by two species of rumen bacteria are reported. One factor caused a marked depression of blood pressure in experimental dogs and sheep and the other produced a rise in blood pressure. The role of these factors in naturally occurring rumen dysfunctions is not known.

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Correction

The following correction should be made in the article by Louis DS. Smith, Ph.D., and Hadleigh Marsh, D.V.M., in the July, 1953, issue (p. 408, second column, third paragraph, fourth line):

It had *not* been treated with trypsin in the course of the preparation.

The Pathology of Experimental and Natural Cases of "Yellow Fat" Disease in Swine

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Literature Cited

In their investigational work on the nature, cause, and prevention of "yellow fat" disease or "steatitis" in mink, Gorham *et al.*[1] showed that mink kits fed diets made up principally of fresh ocean fish scrap had an accumulation of acid-fast pigment in the adipose tissue. When the diets were supplemented with adequate levels of vitamin E, such changes were absent. This work was based on the investigations of Dam,[2] Mason *et al.*,[3] and Granados and his co-workers,[4] whose experiments with rats showed that two nutritional factors, excessive amounts of highly unsaturated fatty acids and inadequate amounts of tocopherols, are concerned in the production of acid-fast pigment in the adipose tissue. It has been demonstrated that the pigment consists of fat soluble and fat insoluble fractions, and that the latter manifested the acid-fast staining characteristics.[3] Evidence indicates that the addition of vitamin E to a diet rich in unsaturated glycerides acts as an antioxidant, thus enabling fat cells to incorporate and stabilize unsaturated fatty acids as "storage" fat.[3]

While the condition in swine has been observed by meat inspectors for many years, little attention has been given to the microscopic changes present in the altered fat. Norwegian investigators[5] noted a yellowish discoloration of the tissues in hogs experimentally fed fat from fish or other marine animals. Hummon[6] described an instance in which pigs that were receiving the waste of a mink feed containing a high proportion of fish scrap were condemned at slaughter because of a "peculiar yellow color of the fat." It was of interest to learn that yellow fat disease also occurred in mink receiving the same feed. Keown,[7] while engaged in federal meat inspection at Vancouver, B. C., noted the Chinese practice of feeding refuse from salmon canneries to swine resulted in their condemnation because of yellowish discoloration of the fat, with a fishy odor of the flesh. Feeding other substances containing highly unsaturated glycerides, such as cod-liver oil[8] and flaxseed,[9] have been shown to produce yellow fat in swine.

Having in mind the experimental work in rats previously mentioned and with their own observations with the disease in mink, Gorham and his co-workers[1] believed that a syndrome was occurring in swine which was similar, if not identical, to yellow fat disease in mink. Consequently, in two controlled experiments, feeding in the first a diet similar to the one used in producing the condition in mink and supplementing the same diet with vitamin E in the second, they summarized their findings as follows: "Young pigs placed on diets containing high percentages of fish scrap developed an acid-fast pigment in the adipose tissue and, in pigs receiving supplemental tocopherol, the deposition of pigment was reduced." It is probable that if higher levels of vitamin E had been used, acid-fast pigment formation would have been prevented.

The purpose of this paper is to elaborate on the pathological findings in the adipose tissue of the experimental swine, as well as to record our findings in tissue sections of naturally occurring cases.

Materials and Methods

Tissues from 10 experimental pigs and 4 naturally occurring cases were studied microscopically. Material from the experimental animals consisted of the internal organs and depot fat, whereas only adipose tissue was made available from the natural cases. It should be mentioned that 9 of the 10 pigs on the experimental diet without vitamin E supplement had, in addition to a yellow discoloration of the fat, a perceptible fish odor of the flesh at the time of disposal. The animals made poor weight gains, had rough coats, lassitude, and pale mucous membranes. The tenth pig died after twenty-one days of an intestinal disorder and apparently had not been on the high fish diet long enough for gross tissue alterations to occur.

Tissues from the 4 natural cases representing 37 affected hogs in four different lots were received from federally inspected abattoirs in San Francisco and Los Angeles, Calif.; Spokane, Wash.; and Portland, Ore. In all instances, the inspectors reported a brownish yellow discoloration of the body and visceral fat with an associated fish-like odor of the tissues. The visceral organs generally showed little or no gross alteration. Their condition was reported as fair to good, but in one lot of 6 affected hogs, 2 were reported as being emaciated. The hogs in two lots were garbage-fed; one lot was fed a ration consisting of one-third

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cooked fish meal and two-thirds grain, but no mention was made of the ingredients fed to the other lot.

All the tissues were fixed in 10 per cent formal-saline solution. Paraffin sections were stained with hematoxylin and eosin and with Verhoeff's carbol-fuchsin by the acid-fast technique. The acid-fast pigment was recorded as trace, +, ++, and +++, according to the relative amounts present in the tissues.

Fig. 1—Section of the pericardial fat from a natural case of yellow fat disease in swine, showing deposition of foreign oily substance lying free in the interstices of the normal adipose tissue. Hematoxylin and eosin stain. x 180.

MICROSCOPIC FINDINGS

The most constant and perhaps significant finding in the yellow fat in both the experimental and natural cases was the deposition of yellowish pigmented fatty material in the interstices of the subcutaneous visceral adipose tissues. This deposition appeared as fine droplets or, quite frequently, as larger discrete globules in groups or islets of variable size. In hematoxylin and eosin-stained sections, the oily deposits appeared pale yellow to golden yellow and, for the most part, were contained within phagocytic cells which not infrequently fused to form foreign-body type giant cells. In the natural cases, however, the fat globules were lying free in the interstices without accompanying phagocytic activity (fig. 1). In areas where the islets accumulated in greater degree, there was compression and sometimes indentation of the adjacent fat cells. Occasionally,

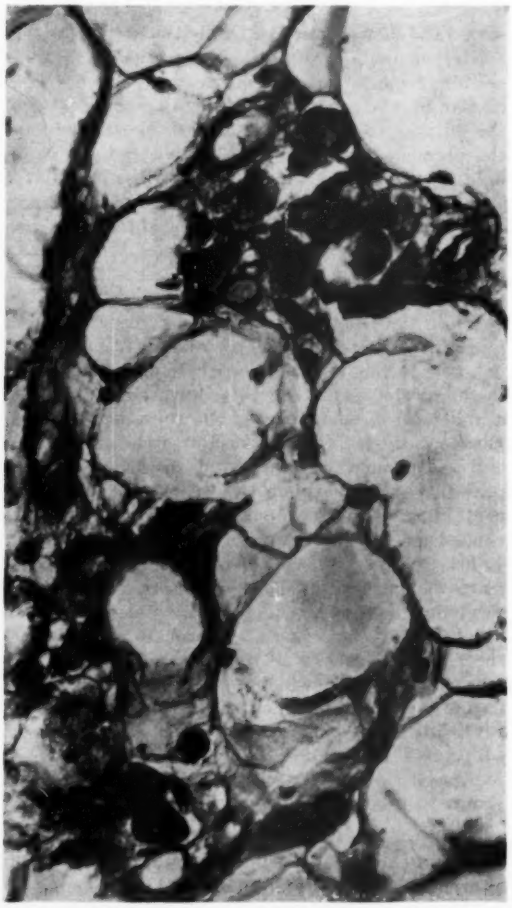

Fig. 2—Section of back fat from an experimental hog showing irregular dark-staining acid-fast deposits in the supporting connective tissue spaces of the normal fat cells. Some of the acid-fast pigment is contained within phagocytes. Carbol-fuchsin hematoxylin stain. x 450.

Fig. 3—Section of back fat from a natural case of yellow fat disease in swine, showing marked focal accumulations of irregular dark-staining deposits of acid-fast pigment. There was no phagocytic activity in this case. Carbol-fuchsin hematoxylin stain. x 450.

the foreign fat globules were seen within the adipose tissue. This was interpreted as permeation into the normal storage fat rather than a disturbed metabolic process occurring primarily in the normal fat cells. Now and then, in areas of slight deposition, the droplets were seen immediately about capillaries or arterioles in the connective tissue spaces. There was no microscopic evidence of necrosis of the normal adipose tissue, although in several cases a few scattered focal accumulations of round cells and polynuclear leukocytes were noted associated with the oily deposits. This cellular reaction, however, was minimal. Fibrosis was lacking.

When the tissue sections were stained by the acid-fast technique many, but not all, of the fat globules and smaller droplets were shown to possess acid-fastness (fig. 2 and 3). The color varied from pink to deep red. Some of the deposits stained only at the periphery. The acid-fast pigment is considered to be the nonsoluble fraction of the highly unsaturated fatty acids in the experimental diets capable of producing the yellow discoloration of the adipose tissue. In 9 of the 10 experimental hogs and the 4 natural yellow fat cases, the pigment was present to a +++ degree, the staining density being variable. In the

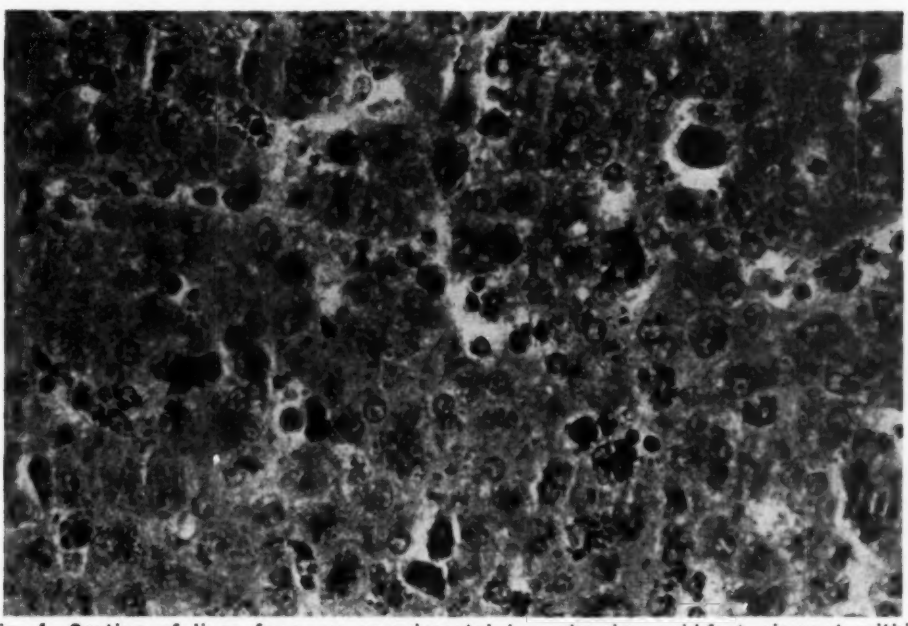

Fig. 4—Section of liver from an experimental hog showing acid-fast pigment within Kupffer cells. Smaller droplets of acid-fast pigment are seen in the cytoplasm of the liver cells. Carbol-fuchsin hematoxylin stain. x 450.

experimental cases, the same degree of pigmentation was present in destroyed hogs as early as thirty-six days and as late as eighty days and in the pigs that succumbed at 37, 40, 50, and 60 days after being fed the experimental diet. One pig that died after twenty-one days of hemorrhagic enteritis was on the high fish diet a sufficient length of time for + acid-fast pigment to form, although the adipose tissue showed no appreciable gross alteration.

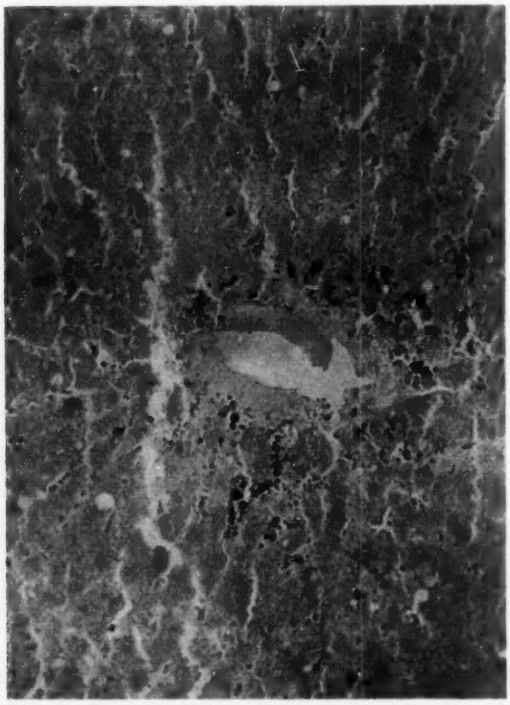

Fig. 5—Section of liver from a natural case of yellow fat disease in swine, showing the concentration of acid-fast pigment in the sinusoids about a central vein. Carbol-fuchsin hematoxylin stain. x 180.

Three pigs on the experimental diet to which vitamin E supplement was added made normal gains and appeared healthy. Two of these animals were destroyed after fifty-four days for autopsy purposes and, although the fat appeared normal and without odor, ++ acid-fast pigment was demonstrated in 1 and a trace in the other. A biopsy of the fat in the third animal was made at the same time, and a trace of acid-fast pigment was found. This pig was continued on a normal diet to be used for food by a student helper. When butchered, this animal showed no gross abnormalities of the fat nor was there any detectable odor. Nevertheless, the meat had to be discarded because of an offensive fish odor that emanated after cooking. It is to be assumed that the amount of vitamin E supplement, while sufficient to maintain the pigs in a healthy condition, did not entirely prevent the formation of acid-fast pigment.

In the animals on the experimental diet without vitamin E supplement, the liver was the only organ to show deposition of acid-fast pigment. The pigment deposits were present in +++ degree and were situated in the Kupffer cells of the sinusoids with greater concentration about the central veins (fig. 4). In addition, the cytoplasm of nearly all the liver cells contained numerous small strongly acid-fast droplets. The pigment gave a negative reaction for iron. There was no associated inflammatory reaction or evidence of cirrhosis. The

Fig. 6—Section of fat about a lymph node from an experimental case of yellow fat disease in swine, showing deposition of acid-fast pigment in the interstices of the normal adipose tissue. There is considerable phagocytic activity and a few giant cells are in evidence. Carbol-fuchsin hematoxylin stain. x 210.

liver from 1 of the natural cases was available for microscopic examination and ++ acid-fast pigment was present in the Kupffer cells but not in the liver cells (fig. 5).

Traces to + acid-fast pigment were found within phagocytic cells in the lymph nodes and spleen in the experimental pigs. Variable degrees of pigment up to +++ were present in the pericardial, perirenal, intrapancreatic, periaortic, and intramuscular fat as well as in the pericapsular fat of the lymph nodes (fig. 6). No degenerative changes of consequence were found in the other visceral organs, including the testes. No evidence of muscular dystrophy or myocardial changes were observed.

SUMMARY

1) "Yellow fat" disease in swine is characterized by a yellow to yellowish brown discoloration of the adipose tissue, usually with a fish-like odor of the flesh.

2) The disease is frequently associated with the feeding of excessive amounts of fish or fish products containing highly unsaturated fatty acids.

3) Microscopically, the yellow fat shows a deposition of foreign fat globules in the interstices of the adipose tissue, some of which show acid-fast characteristics.

4) The addition of vitamin E supplement to a high fish diet has been shown to reduce the formation of acid-fast pigment in an experimental lot of hogs. Perhaps a higher level of vitamin E might have entirely prevented the occurrence of pigment.

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The "Normal" Liver of the Pig; Is It an Example of Purely Portal (and Therefore Subclinical) Cirrhosis?

A Preliminary Report

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EVER SINCE Kiernan [1] published his masterful study on the liver of the pig in 1833, this animal has served as the paradigm of liver architecture. Almost every textbook of human histology bases its account of the liver on that of the pig. Hepatic lobules in the pig, first described by Wepfer in 1664, are separated from one another by walls of connective tissue, thus giving the liver of this species a schematically clear-cut structure. A characteristic textbook statement may be quoted, "The distinctness with which the lobules are defined depends upon the amount of the interlobular tissue. This is notably abundant in the hog's liver, in which the lobules appear as sharply marked polygonal areas. In the human liver, on the contrary, the interlobular tissue is very meagre, the lobules, in consequence, being poorly defined and uncertain in outline." [2] Thus it is assumed that the hepatic lobule is a permanent, structural entity (as, in fact, it is in the hog) which is only indistinct in man because of poorly developed interlobular connective tissue.

It was shown recently [3] that the hepatic lobule in man, dog, rabbit, and mouse is only a temporary expression of blood pressure conditions, that by elevating the blood pressure in the hepatic veins, or by lowering it in the portal vein, the hepatic lobules can be abolished within two minutes and another pattern of lobules centered around portal canals can be produced. The lobules reveal themselves only as temporary expressions of blood pressure distribution, and the liver as a whole appears as a unified continuum of communicating sinusoids and connected laminae.

The liver of the pig, on the other hand, is rigidly subdivided into permanent lobules. Instead of being a paradigm for all mammalian livers, it appears to be an exception. Due to its different structure, pork liver is exceptional also from the standpoint of human consumption. It is tough because of the presence of the connective tissue septums. It is important, therefore, not only from the standpoint of comparative anatomy but also from the standpoint of the meat industry, to attempt to understand this structural divergence.

It will be seen that the pathological approach can supply the understanding. We may ask ourselves whether there exists a pathological condition in human livers which produces a similar liver structure to that of the pig. Such a condition does exist. Cirrhosis is a transformation of the human liver in which the parenchyma is divided into small nodules by septums of connective tissue.

With this parellel in mind, the liver of the pig was studied. It was compared with the human liver in cirrhosis. It was found, indeed, that septation is not the only feature which the pig's liver has in common with the human cirrhotic liver.

MATERIALS AND METHODS

For this study, five pig livers obtained from an abattoir were injected with India ink, one through the hepatic artery, two through the portal vein, and two through a hepatic vein. Well-injected and noninjected blocks of these specimens were fixed by immersion in 10 per cent formalin. To study certain features of the vascular pattern, thick sections were made by hand and cleared in balsam. Other pieces of the same specimen were cut serially at 10 and 30 μ. For reconstruction, serial sections were projected on glass plates and traced in various colors of India ink to distinguish various systems. From the models, consisting of stacked glass plates, drawings were made. In addition, conventional histological methods were employed.

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* All new data on human cirrhosis mentioned in this paper have been presented by Elias and Popper in an exhibit entitled "Functional Morphology of the Normal and Cirrhotic Liver," at the convention of the American Medical Association in Atlantic City, 1951.

COMPARISON OF THE PIG'S LIVER WITH THE HUMAN LIVER IN CIRRHOSIS

General Architecture.—In human cirrhosis, the liver is divided into so-called nodules. These are not identical with the original hepatic lobules of healthy livers. Depending on the initiation of the cirrhotic process, the nodules may be pie-like sectors of hepatic lobules, in cirrhosis following passive congestion, central toxic necrosis, and periportal inflammation, or they may be territories cut out of the continuous hepatic parenchyma in an irregular manner, entirely independent of the original lobular architecture after fatty degeneration and after massive necrosis. As a consequence, the vascularization of the human cirrhotic nodule is entirely different from the normal hepatic lobule. In sectional nodulation (pie-like division), the former central veins come to lie in the connective tissue septums which have come to connect the portal with the central fields. They are therewith eliminated from the parenchyma. Tortuous tracts of former sinusoids take over the function of the efferent veins in the individual nodules. Former sinusoids, having become incorporated in the newly formed septums, are transformed into portaportal and into portahepatic shunts.

In the liver of the pig, the basic architecture is much less drastically altered. It appears that the territories surrounded by septums are still identical with the original hepatic lobules of newborn pigs in which, according to the thorough observations by Johnson,[4] no septation exists as yet. In the adult pig, each walled-in lobule has its typical central vein into which individual sinusoids empty.

The center of the pig's liver lobule is identical in structure with the center of liver lobules in man and most mammals as described by Elias.[5] Hepatic veins that run in the septums are sublobular and collecting veins. The septums in the pig's liver do not connect portal with central fields. They connect portal with portal and portal with sublobular and collecting vein territories. Thus, in regard to the general architecture, the liver of the pig is not entirely identical with the human cirrhotic liver. Yet, except for the smallest hepatic veins, the central veins, the architecture is more similar to the human cirrhotic liver than to the normal human liver. For, while hepatic and portal veins in the normal mammalian, including human, liver run in separate, cylindrical connective tissue trabeculae, in the liver of the pig, as well as in human cirrhosis, they run in the same flat septums.

Portahepatic and Portaportal Anastomoses.—It has been shown by Ungar[6] and by Popper, Elias, and Petty[7] that in human cirrhosis, numerous direct anastomoses connect neighboring portal veins with one another, and that just as many shunts exist between portal and hepatic, mostly former central, veins.

In the adult pig, some, but not numerous, anastomoses between neighboring portal veins but none between portal and central veins can be demonstrated. The anastomoses in the pig are not as frequent as in human cirrhosis. Yet they establish a further similarity.

Destruction of the Limiting Plate and Hypotrophic Septal Cells.—In normal mammalian livers, the portal canals and the adventitiae of the sublobular and larger hepatic veins are surrounded by a continuous sheet of liver cells, one cell thick, called the limiting plate.[8] The limiting plate is continuous with the intraparenchymal liver plates. From the distributing portal veins, short afferent (inlet) venules arise. These penetrate through holes in the limiting plate and supply directly a network of periportal sinusoids. From this periportal or paraportal sinusoidal network, radial sinusoids arise which convey the blood toward the central vein. It thus comes about that the entire network of sinusoids is separated from the portal canal by the limiting plate, except for the inlet venules which are spaced at relatively large intervals. In other words, a continuous layer of liver cells one cell thick intervenes between the periportal connective tissue and the paraportal sinusoids.

If periportal inflammation initiates the cirrhotic process in man, the first part of the parenchyma to be destroyed is the limiting plate. One finds, in early stages, intralobular liver plates ending individually and abruptly at the boundary of a portal canal. The sinusoids come in contact with the connective tissue of the portal canal.

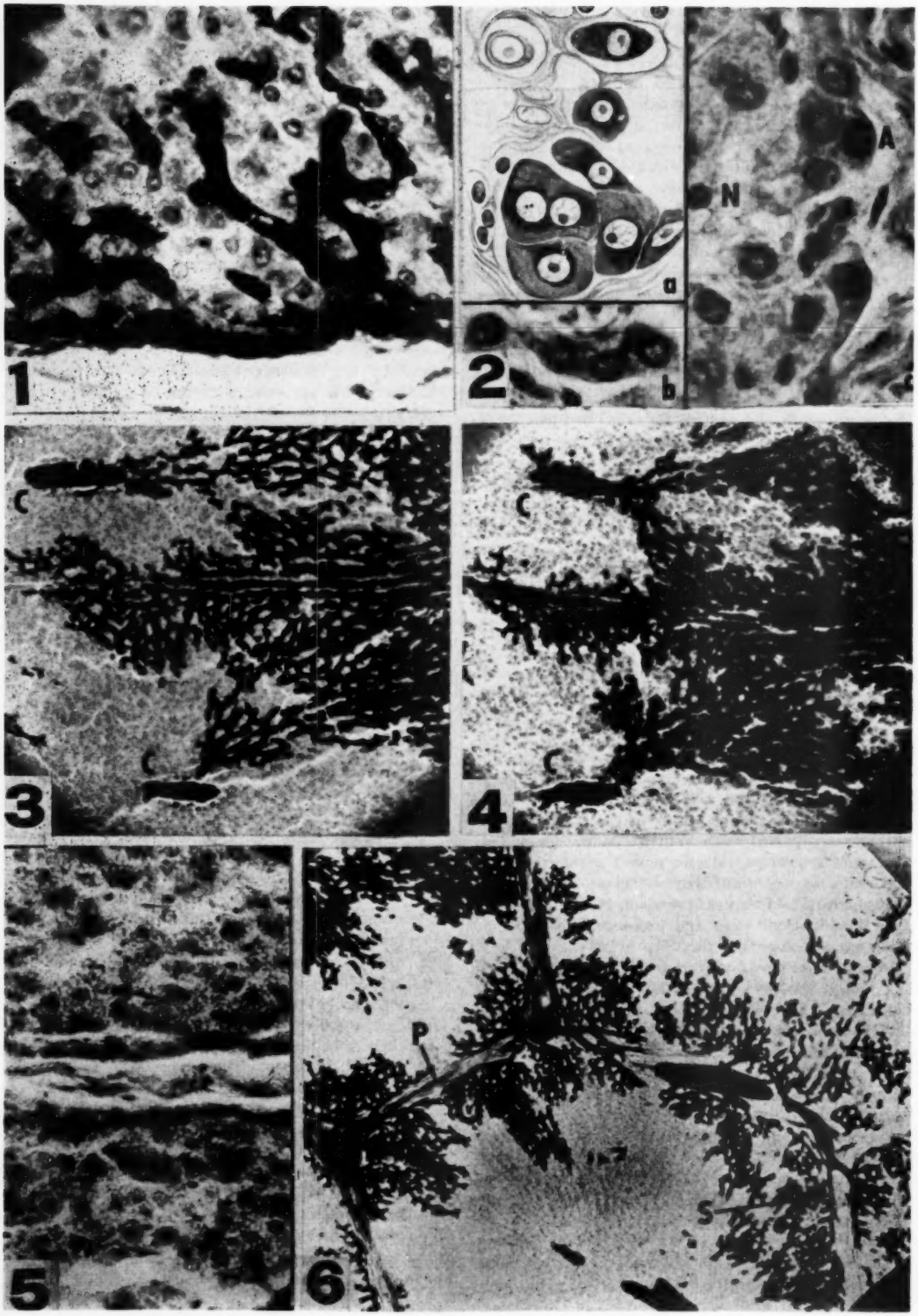
In advanced cases of human cirrhosis, one finds small, flattened hypotrophic or atrophic, dark staining liver cells in the septums very close to the parenchyma. These cells may occur singly, in small groups, or arranged in larger sheets. In some cases, they appear to have become detached from the parenchyma. In other cases, it may be assumed that they have become incorporated in developing septums.

In the adult pig, the limiting plate is absent at most places. The limiting plate is present in newborn pigs but is destroyed at a later date; the age at which this occurs is not yet known. In specimens injected with India ink one can see paraportal sinusoids which are flanked on one side by connective tissue, *i.e.*, on that side where normally the limiting plate would be found (fig. 1).

Occasionally long sheets of liver cells are seen at the periphery of a septum. These could be mistaken for limiting plates. However, closer inspection at high magnification (fig. 2), reveals that one is dealing here with the same kind of atrophic liver cells which are encountered in human cirrhosis. Also, small groups of such atrophic cells can be seen (fig. 2).

Examining the general architecture and vasculature of the liver of the adult pig, a certain similarity to human cirrhosis could be found as we have seen above. The presence of the atrophic cells and the absence of the limiting plate strongly leads one to suspect the condition found in the pig's liver as being cirrhosis.

Formation of Septums.—The formation of sep-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2
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tums is varied in human cirrhosis. Among several mechanisms one mode of septum formation may be reported here because of its similarity to swine septum formation.

Whenever mechanical traction is exerted on a portion of human liver tissue, the sinusoids and liver plates in that territory are stretched, straightened out, and flattened. In human livers this may occur in the neighborhood of a collapsing portion of liver after massive necrosis, or it may occur within existing cirrhotic nodules as a result of vigorous expansion of the nodule in the process of regeneration. The stretching and flattening of sinusoids and liver plates result in pressure atrophy and subsequent death of the liver cells composing the stretched plates. An empty, flat fissure appears in their stead. Subsequently, the fissure is filled out with connective tissue and a septum is formed. Some of the old sinusoids may persist in the septum. Since they no longer run between liver cells, they are now shunts between the vessels which they formerly connected. Usually, some of the liver cells survive. These remain in a septum as the above described flat, dark-staining, atrophic cells.

While the first formation of septums in young pigs was not observed in the present study, new formation of additional septums in adult pigs was observed. Johnson [4] reported that not every lobule of the pig's liver is completely surrounded by connective tissue, but that there are groups of lobules incompletely separated. Establishment of septums between these lobules could be observed in the present study. The process is identical to the one described above for human cirrhosis.

Figure 3 shows a tract of sinusoids (injected with India ink through the portal vein) which are straightened out. Most of their cross connections have disappeared. Between them stretched, flattened liver plates can be seen. Following the same region in serial sections, one comes to a place in which the stretching process has not yet gone so far (fig. 4). At this location, sinusoidal connections between two central veins (a condition normal to most mammalian livers) can still be seen going through the stretched territory. Following the series in the opposite direction one encounters a level where most sinusoids and some liver plates have disappeared (fig. 5). A fissure lined by two flattened, atrophic liver cell sheets can be seen. These sheets formerly were within the undivided parenchyma. Hence, they are not limiting plates. They are those atrophic cell sheets which were described above. Still lower in the series of sections one can see the beginning of connective tissue deposition in the fissure. When a new septum is formed, most of the sinusoids of the area disappear. One layer of sinusoids may persist in the center. These centroseptal vessels become portaportal anastomoses (fig. 6P, 7P'); however, the marginal sinusoids remain. These become paraseptal sinusoids and are supplied with portal blood. In this fashion, a pattern develops very similar to the terminal twigs type 2 as described by Elias.[5] Figure 6 illustrates this pattern at various places (P'). In figure 3, one can identify

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Fig. 1—Peripheral part of a liver lobule of a pig, injected with India ink through the portal vein. The paraseptal sinusoids are in immediate contact with the septum (below). No limiting plate intervenes between the septum and the network of sinusoids (first evidence of cirrhosis).

Fig. 2—Atrophic liver cell groups in and near a portal canal (second evidence of cirrhosis). Fig. 2a shows a few atrophic liver cells in a septum of a human liver affected with Laennec's cirrhosis. These are stained with Mallory and drawn with the brush; therefore, they have a slightly different appearance from the atrophic cells from a septum of a pig's liver seen in figures 2b and 2c. If one takes into consideration the differences in the nuclear stain, the similarity of the cells from both cases is remarkable. There is a greater affinity for both basic and acid stains in the cytoplasm. One may compare the atrophic cells (A) with the normal liver cells (N) in figure 2c.

Fig. 3—Liver of pig injected with India ink through the portal vein. A pattern of traction has straightened out intralobular liver plates and sinusoids. The stretched and flattened liver plates will undergo necrosis. First a fissure and then a septum will appear in their stead (see fig. 5). The middle one of the sinusoids may become a portaportal anastomosis. The other two straightened sinusoids will become paraseptal sinusoids, as shown in fig. 1.

Fig. 4—Another section of the same region as shown in fig. 3. In this section some of the old sinusoids connecting the two central veins (c,c) can still be seen.

Fig. 5—Another section of the same region. The middle liver plates have already disappeared at this level. A fissure is found in their stead. A sinusoid in the process of transformation into a portaportal shunt occupies the center of the fissure. Connective tissue begins to accumulate about this vessel. Two atrophic, stretched liver plates are flanking the fissure. In the fissure, a connective tissue septum will develop.

Fig. 6—Thick section from the same specimen showing the mode of terminal branching of the portal veins, and two portaportal shunts. P = portaportal shunt; S = paraseptal sinusoids. Identical structures, unlabeled, are seen at other locations.

those sinusoids which will become the centroseptal, portaportal anastomoses and the paraseptal sinusoids.

Flexible structures such as sinusoids and liver plates can only be straight or plane if they are under tension. If no great tensile force acts upon them, they are crooked. The latter is the normal condition in the interior of lobules. The straightening and flattening described above must, therefore, be ascribed to tension. What is the cause for this tension? Tension can exist under two conditions: (1) active contraction of the straightened object, and (2) traction exerted from without. If the liver plates and sinusoids of this region contracted, they should become thicker. Since they are thinner than normal, active contraction must be ruled out. External traction remains. What can be the source of the external pulling? It must be sought in the growth and expansion of the lobules. In figure 8, evidence of the intense growth is presented. A tract of septums (S) limiting the area in which the traction pattern is

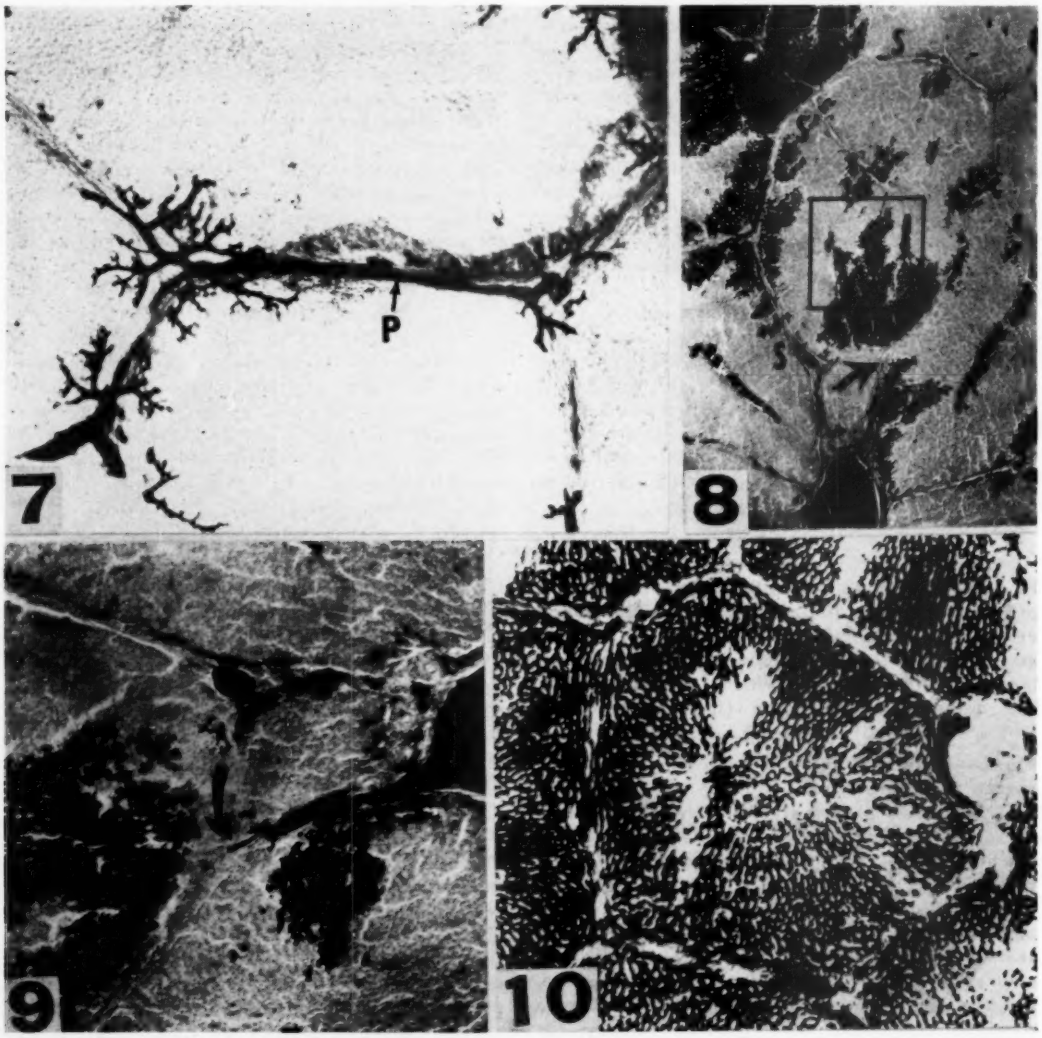

Fig. 7—A portaportal shunt (P). Thick section.

Fig. 8—A curved tract of septums is shown as evidence of the expansion of the territory on its concave side. The rectangle marks the area of figures 3, 4, and 5. The arrow identifies the location of a kink in a septum, more highly magnified (and taken 60 μ above this level) in fig. 9.

Fig. 9—Kink in a septum produced by traction exerted by stretched liver plates and sinusoids (left).

Fig. 10—Early stage in the subdivision of a growing lobule by traction, a process identical to the one shown in figures 3, 4, and 5.

found (rectangle, fig. 3) is smoothly curved and concave toward that area. It is obvious from this distortion of the tract of septums that the portion of liver on its concave side is expanding. More evidence of traction is found in figure 9 from the same area where one sees a septum kinked by the traction exerted by the stretched plates and sinusoids. Expansion of parenchyma that is walled in by septums is a common occurrence in human cirrhosis, and it is generally considered to be a regenerative response to destruction of liver tissue. In the case of the pig, destruction of the limiting plate and of paraseptal liver cells may be the stimulus for such expansive growth.

Such division of lobules by traction is found frequently in the pig's liver. Figure 10 gives another illustrative example of this process.

DISCUSSION

It is clear then that the liver of the pig can not be considered to be normal.* Particularly, the absence of the limiting plate marks it as atypical. The fact, reported by Johnson,[4] that at birth the liver of the pig is not lobulated by septums makes it probable that the strict lobulation found in adult pigs is not necessarily typical even for the pig.

Comparison with the human liver shows that adult pigs exhibit a definite cirrhosis; however, it is a mild form of cirrhosis. It is mild because portahepatic anastomoses are absent and because the hepatic veins are not flattened to such a degree as they are in many cases of human cirrhosis.[5] It has been suggested** that the flattening of the hepatic veins causes the obstruction to blood flow in the human cirrhotic liver and thus is the source of portal hypertension. The reason for the flattening of the hepatic veins, according to Kelty, Baggenstoss and Butt,[5] is the vigorous expansion of numerous regenerating nodules. In the pig, such regenerative expansion occurs, as we have seen. It occurs, however, only sporadically. The straightness of the vast majority of septums demonstrates that the intralobular pressure is usually moderate. Thus, no permanent flattening of hepatic veins occurs.

In human cirrhosis, due to the frequency of portahepatic and arteriohepatic anastomoses, most of the blood is shunted past the parenchyma. As a consequence, almost every nodule, after a time of vigorous, regenerative growth, undergoes central necrosis. In many nodules, central necrosis begins at a time when many cell divisions are still under way near the periphery of the nodules. Another factor promoting central necrosis is the stagnation of blood due to the flattening of the hepatic veins. In the pig, there is no central necrosis because of the absence of portahepatic shunts and because of the persistent patency of the hepatic veins. The reason for this difference seems to be the fact that the connective tissue proliferation proceeds from the portal canals and while it encompasses the territories of the sublobular and larger hepatic veins, connective tissue proliferation does not occur in the neighborhood of the central veins. Therefore, the chances for sinusoids to develop into portahepatic shunts are poor.

What is the evidence for the portal origin of the septums? It is the fact that the traction fissures appear exactly in the middle between two central veins in the area which Pfuhl[9] called "septum vasculare." This area is that part of the sinusoidal network that is supplied by the terminal ends of portal veins. The septums vascularia connect neighboring terminal portal branches.[5]

SUMMARY

The liver of the pig, characterized by sharply defined lobules separated from each other by connective tissue septums, exhibits a form of portal cirrhosis of unknown etiology. The following would lead one to identify the condition as cirrhosis because of their similarity to human cirrhosis: (1) portal and hepatic veins run in the same septums, (2) occasional porta-portal anastomoses occur, (3) the limiting plate is missing, (4) atrophic liver cells, characteristic for many forms of human cirrhosis, occur in the septums, and (5) new septums are formed under the influence of mechanical traction exerted on certain parts of the parenchyma.

Swine cirrhosis is benign, since portahepatic shunts are absent. As a consequence, no central necrosis takes place. In the absence of central necrosis, vigorous regenerative expansion of nodules occurs sporadically. Therefore, no permanent

* The word "normal" should be taken *cum grano salis*, for it appears to be normal for the pig to have an abnormal mammalian liver. Prof. Jean Oliver once said, "The lung in pneumonia is not abnormal, for it is normal for the lung to react to pneumococci in this fashion."

** Conference on Liver Injury, Macy Foundation, May-June, 1952.

flattening of hepatic veins takes place, and in turn, portal hypertension does not develop.

Cirrhosis in man [7] and experimental cirrhosis in the rat [6,10] is portahepatic or hepatoportal. In the pig, we find a purely portal type of cirrhosis.

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Ovine and Porcine Penicillin Blood Levels Obtained with Parenteral Dibenzylethylenediamine Dipenicillin G

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THE MAINTENANCE of penicillin blood levels without frequent injection is important in veterinary practice because the cost in time and labor of repeated administration may limit the extent of therapy.

The intramuscular administration to human beings of the new, repository type of penicillin, N,N'-dibenzylethylenediamine dipenicillin G, has resulted in penicillin blood levels lasting for two weeks and longer.[1] Consequently, much interest has been attached to investigations of the duration of penicillin blood levels following administration of this new compound to animal species of veterinary interest.

The chemistry and pharmacology of this compound have been reported,[2-4] and blood levels following oral administration to dogs have been determined.[5] Most interest, however, has centered around the duration of blood levels following single intramuscular injection of dibenzylethylenediamine dipenicillin G to cattle,[6] dogs,[7] and turkeys.[8] This report of levels following its intramuscular administration to sheep and swine extends the data to include all of the species of major economic importance.

Dr. Kral is from the Department of Medicine, Veterinary Hospital, University of Pennsylvania; Drs. Huebner and Blumner are from the Department of Veterinary Service, Wyeth Laboratories, Philadelphia, Pa.

An expression of gratitude is extended to Dr. Wm. F. Elias, Mr. H. J. Merrion, and Miss Aida Seltzer of the Antibiotic Division, Wyeth Laboratories, West Chester, Pa., for their technical assistance in this project.

* Injection bicillin ® supplied by Wyeth Laboratories, Division of American Home Products Corporation, Philadelphia.

To determine the response elicited by dibenzylethylenediamine dipenicillin G* in normal sheep and swine, single intramuscular injections in aqueous suspension were administered. Prior to injection, the serum was examined and found to be normal and negative for penicillin-like activity. For purposes of comparison, procaine penicillin G, similar in amount and preparation, was administered to control animals at the same time and under identical conditions. Blood samples were withdrawn at the postinjection times indicated in the tables. The serum content of penicillin was determined by the microbiological cup plate method.

The sheep weighed from 110 to 125 lb. and received 300,000 units of either procaine penicillin G or dibenzylethylenediamine dipenicillin G (tables 1, 2, and fig. 1).

During the course of the experiment, all sheep were inspected and examined physically at regular intervals. Hemoglobin determinations, red and white cell counts, and fecal examinations were made. No significant changes were noticed in

TABLE 2—Blood Serum Levels of Sheep Receiving 300,000 Units of Procaine Penicillin G in Aqueous Suspension per Cubic Centimeter—Control for Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension

Sheep	Control	Units of penicillin per milliliter of blood serum					
		1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	3 days
1X	0	>1.000	0.200	0.640	0	0	0
2X	0	0.390	0.710	0.740	0.450	0.098	0
Ave.		0.695	0.455	0.690	0.225	0.049	0

TABLE 1—Blood Serum Levels of Sheep Receiving 300,000 Units of Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension per Cubic Centimeter

Sheep	Weight (lb.)	Control	Units of penicillin per milliliter of blood serum									
			1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	3 days	4 days	5 days	6 days	7 days	8 days
1	125	0	0.074	>1.000	0.064	0.144	0.023	0.012	0.019	0	0	0	0
2	122	0	0.086	0.110	0.240	0.051	0.033	0.012	0.010	0	0	0	0
3	123	0	0.047	0.068	0.062	0.035	0.026	0.022	0.022	0.020	0.013	0.024	0
4	125	0	0.011	0.041	0.027	0.038	0.044	0.042	0.027	0.017	0	0	0
5	115	0	0.062	0.062	0.039	0.039	0.046	0.041	0.035	0.043	0.019	0.024	0
6	124	0	>1.000	0.045	0.040	0.047	0.024	0.036	0.021	0.013	0	.....	0
7	118	0	0.470	0.108	0.213	0.061	0.062	0.035	0.012	0	0	0	0
8	122	0	0.305	>1.000	0.036	0.037	0.050	0.033	0.025	0.062	0.020	0	0
9	115	0	0.820	>1.000	0.043	0.036	0.040	0.020	0.011	0	0	0	0
10	115	0	>1.000	0.025	0.031	0.026	0.024	0.018	0.018	0.017	0.011	0.010	0
11	120	0	>1.000	0.032	0.031	0.051	0.052	0.044	0.025	0.021	0	0	0
12	110	0	>1.000	0.039	0.034	0.046	0.062	0.034	0.018	0.011	0	0	0
Ave.			0.489	0.294	0.072	0.051	0.040	0.029	0.020	0.017	0.005	0.005	0

TABLE 3—Sheep Blood Hemoglobin Content, Red and White Blood Cell Count

Sheep (No.)	Hb.—Gm./100 ml.			r. b. c.—10^6/mm.^{3}			w. b. c.—10^3/mm.^{3}		
	A	B	C	A	B	C	A	B	C
1	11.5	14.3	14.0	10.48	13.80	13.75	17.50	11.3	9.12
2	10.7	10.2	8.5	10.86	9.30	9.12	11.60	16.5	8.50
3	10.1	11.0	9.4	11.31	10.41	8.80	12.00	10.0	11.30
4	10.5	10.5	10.6	10.08	11.74	12.35	7.40	18.35	14.50
5	10.1	10.0	10.7	9.43	10.68	12.20	9.00	5.6	11.05
6	11.5	11.3	10.5	9.74	12.51	12.85	10.35	12.55	12.95
7	8.7	8.6	9.7	7.78	9.33	10.10	12.30	15.50	9.50
8	11.9	11.8	12.2	11.64	12.47	12.10	16.15	9.15	13.75
9	12.2	12.0	12.3	11.47	11.13	13.15	12.10	16.25	10.60
10	10.9	10.9	13.3	10.16	10.27	13.20	8.10	5.75	8.80
11	9.0	8.1	7.0	7.38	6.84	8.70	15.70	7.55	13.30
12	10.4	10.3	10.7	9.73	9.67	13.36	20.10	18.5	31.15
Ave.	10.625	10.75	10.74	10.00	10.65	11.64	12.7	12.3	12.9

A—Preinjection determinations; B—six days postinjection determinations; C—eleven days postinjection determinations.

TABLE 4—Blood Serum Levels of Hogs Receiving 200,000 Units of Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension per Cubic Centimeter

Hog	Weight (lb.)	Control	Units of penicillin per milliliter of blood serum							
			1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	3 days	4 days	5 days
1	62	0	0.198	0.045	0.075	0.039	0.040	0	0	0
2	64	0	0.180	0.099	0.092	0.082	0.205	0	0	0
3	60	0	0.512	0.170	0.153	0.078	0.022	0	0.071	0
4	62	0	0.257	0.150	0.148	0.062	0.045	0.052	0	0
5	68	0	0.223	0.199	0.158	0.050	0.025	0	0	0
6	68	0	0.208	0.128	0.088	0.084	0.055	0	0	0
7	68	0	0.163	0.084	0.074	0.082	0.076	0	0	0
8	66	0	0.108	0.050	0.044	0.047	0.102	0	0	0
9	62	0	0.148	0.150	0.074	0.057	0.089	0	0	0
10	80	0	0.140	Discontinued						
11	68	0	0.132	0.064	0	0.047	0.068	0	0	0
12	68	0	0.450	0.168	0.141	0.112	0.082	0.034	0	0
Ave.			0.227	0.119	0.095	0.067	0.074	0.008	0.007	0

TABLE 5—Blood Serum Levels of Hogs Receiving 200,000 Units of Procaine Penicillin G in Aqueous Suspension per Cubic Centimeter—Control for Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension, 200,000 Units per Cubic Centimeter

Hog	Weight (lb.)	Control	Units of penicillin per milliliter of blood serum							
			1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	3 days	4 days	5 days
14	69	0	>1.000	0.048	0	0.028	0	0	0	0
15	72	0	>1.000	0.780	0.880	0.028	0	0	0	0
Ave.			>1.000	0.414	0.440	0.028	0	0	0	0

TABLE 6—Blood Serum Levels of Hogs Receiving 300,000 Units of Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension per Cubic Centimeter

Hog	Weight (lb.)	Control	Units of penicillin per milliliter of blood serum								
			1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	168 hrs.
1	80	0	0.054	0.430	0.045	0.037	0.029	0.037	0.045	0.028	0.103	0
2	75	0	0.065	0.079	0.047	0.062	0.062	0.046	0.090	0.062	0.055	0
3	85	0	0.053	0.038	0.042	0.054	0.054	0.059	0.058	0.057	0.082	0
4	60	0	0.073	0.064	0.060	0.046	0.096	0.062	0.059	0.061	0.050	0.026
5	70	0	0.062	0.062	0.066	0.062	0.049	0.062	0.049	0.034	0.022	0
6	105	0	0.136	0.152	0.131	0.076	0.066	0.079	0.023	0	0	0
7	55	0	0.185	0.131	0.145	0.100	0.079	0.070	0.022	0	0	0
8	75	0	0.068	0.150	0.109	0.096	0.046	0.029	0.024	0.019	0	0
Ave.			0.087	0.138	0.081	0.067	0.060	0.056	0.046	0.033	0.039	0.003

TABLE 7—Blood Serum Levels of Hogs Receiving 300,000 Units of Procaine Penicillin G in Aqueous Suspension per Cubic Centimeter—Control for Dibenzylethylenediamine Dipenicillin G in Aqueous Suspension, 300,000 Units per Cubic Centimeter

Hog	Weight (lb.)	Control	Units of penicillin per milliliter of blood serum									
			1 hr.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	168 hrs.
9	85	0	0.700	0.420	0.280	0.130	0	0	0	0	0	0
10	80	0	0.260	0.455	0.178	0.185	0.130	0.026	0	0	0	0
Ave.			0.480	0.438	0.229	0.158	0.065	0.013	0	0	0	0

these animals during the course of observation (table 3).

The swine weighed from 55 to 105 lb. One group received 200,000 penicillin units of either procaine penicillin G or dibenzylethylenediamine dipenicillin G. Similarly, a second group received 300,000 units of either compound (tables 4, 5, 6, 7, and fig. 2).

A comparison of tables 4 and 6 indicates, apparently, that an increase in the dose of dibenzylethylenediamine dipenicillin G results in an increase in the duration of penicillin blood levels.

This observation has also been made on human beings, dogs,[7] and turkeys.[8] However, there seems to exist a species difference in this respect, since an increase in the dose given to cattle produces higher penicillin blood levels without an increase in their duration.[9]

SUMMARY

The administration of a single intramuscular injection of 300,000 units of dibenzylethylenediamine dipenicillin G to sheep weighing from 110 to 125 lb. resulted in assayable blood levels persisting for five to seven days. Similar injections of procaine penicillin G resulted in serum levels lasting for twenty-four to forty-eight hours.

Similarly, the administration of a single intramuscular injection of 200,000 units of dibenzylethylenediamine dipenicillin G to swine resulted in assayable blood levels persisting for two to four days. Injections of procaine penicillin G resulted in blood

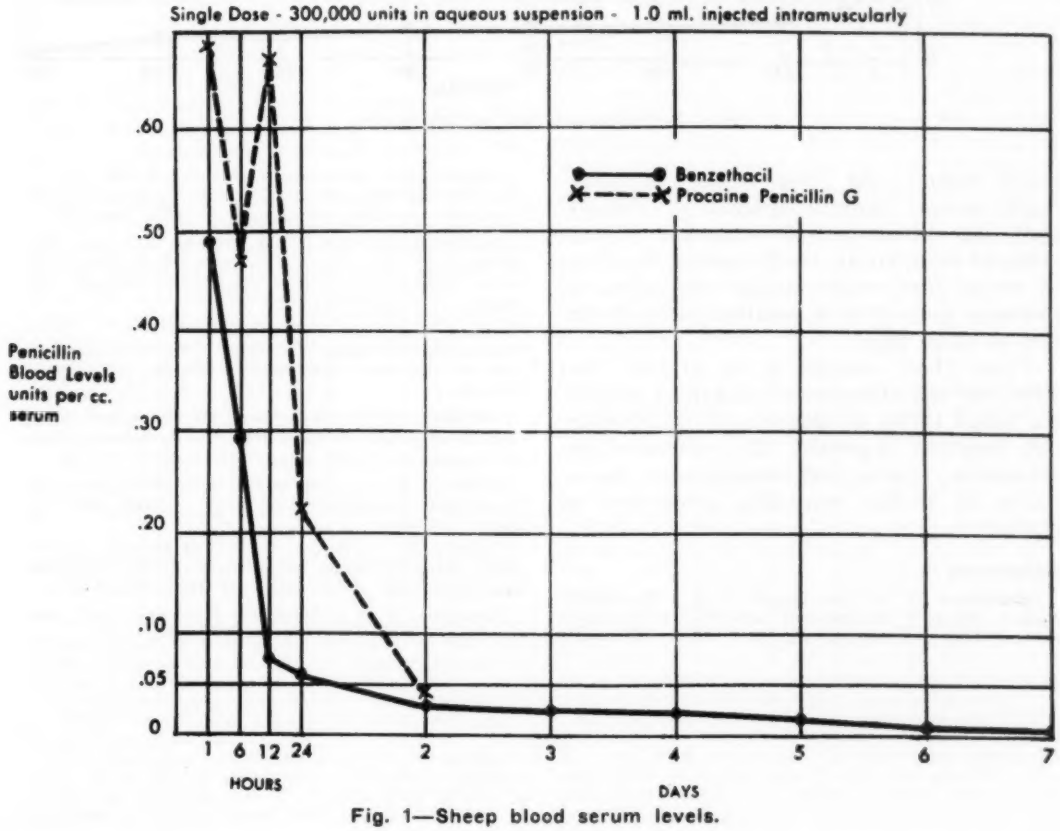

Fig. 1—Sheep blood serum levels.

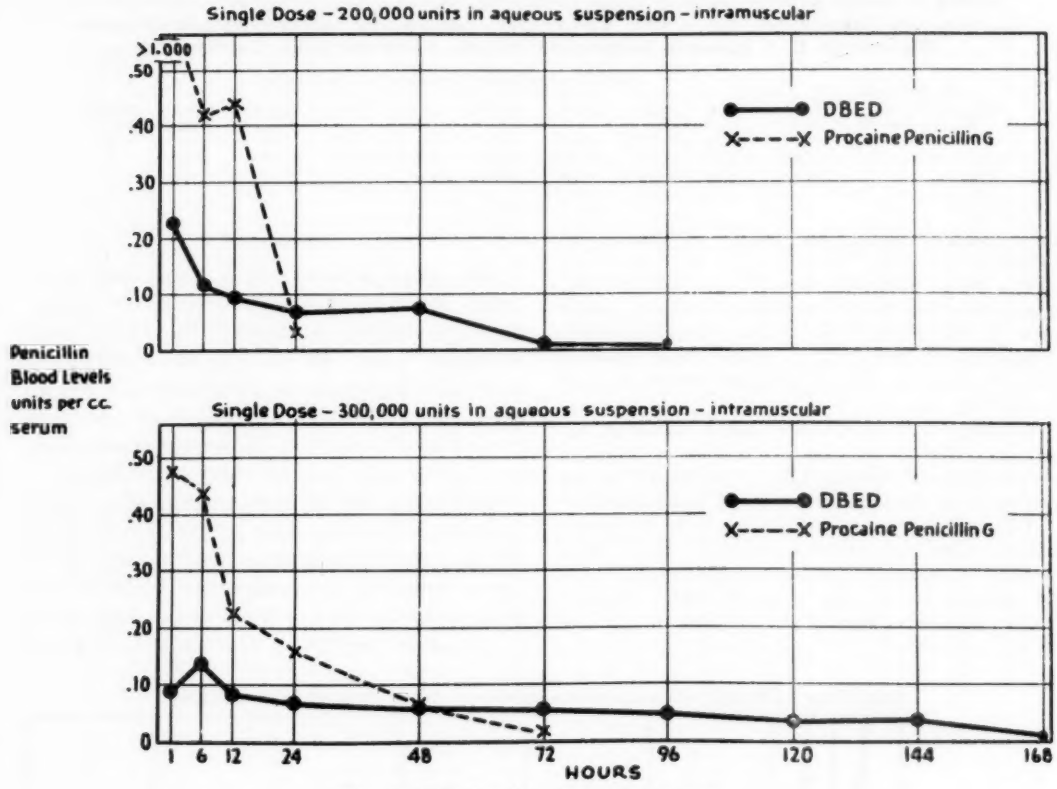

Fig. 2—Porcine blood serum levels.

levels lasting for twenty-four to forty-eight hours. Administration of 300,000 units of dibenzylethylenediamine dipenicillin G resulted in levels lasting for four to seven days while similar injections of procaine penicillin G resulted in levels for two to three days.

From these results, it is obvious that when the maintenance of sustained penicillin blood levels in sheep and swine, without frequent injection, is a primary consideration, dibenzylethylenediamine dipenicillin G is the penicillin compound of choice.

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Giardiasis in the Chinchilla. I. Observations on Morphology, Location in the Intestinal Tract, and Host Specificity

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IN RECENT years the chinchilla has taken a prominent place among the fur-producing animals of the United States. This rapid development of the industry and the high economic value of the chinchilla, which has greatly limited their use for experimental studies, are primarily responsible for the dearth of information regarding the animal and its diseases.

LITERATURE CITED

A number of workers have attempted to differentiate the species of Giardia by morphological variations shown by the trophozoite stage found in the different hosts.[1-3] Tsuchiya [4] cast some doubt on the validity of this method of differentiation. Many attempts have been made to transmit the parasite from one host to another.[5-8] Nothing more than a very temporary infection resulted from any of these attempts.

MATERIALS AND METHODS

One ward containing 107 cages, or approximately 214 animals, of the Evergreen Chinchilla Ranch, Columbus, Ga., was used for the purpose of surveying the incidence of Giardia infection. The 18 animals which were made available for experimental purposes were housed in wire-bottom cages in an air-conditioned ward. They were fed and cared for, except in a few special instances which will be explained later, in a manner similar to those kept at the commercial ranch.

All diagnostic examinations for the presence of Giardia cysts were made by using either the direct fecal smear or the standard zinc sulfate concentration technique. The direct smears were prepared using polyvinyl alcohol fixative solution as recommended by Brooke and co-workers.[9] After overnight drying, the smears were stained with chromotrope light green stain as used by Wheatley.[10] The technique used for collection and concentration of clean Giardia cysts was a slight modification of the technique developed by Rendtorff [11] of the United States Public Health Service.

Three animals were killed for the purpose of studying the localization of Giardia organisms in the intestinal tract. Immediately after death the intestinal tract was removed, and ligations of the small intestine were made at 4-in. intervals. Representative samples were taken from the mucosa and combined with 2 drops of polyvinyl alcohol fixative on a clean slide. A smear preparation was made and stained in the same manner as previously described for the fecal smears.

MORPHOLOGY

Morphological studies were made of the Giardia species from the chinchilla in an attempt to establish the relationship of this organism to other described species of Giardia. The trophozoite stage appears as a bilateral symmetrical flagellate which tapers sharply toward the posterior end. Two heavy-staining axonemes originate approximately 3 μ from the anterior end and extend on a longitudinal plane to the posterior end. From each of these axonemes a caudal flagellum extends posteriorly. Two pairs of flagella originate from the anterior blepharoplasts. The anterior pair appear to cross each other in the cytoplasm and pass to the anterior end of the organism on the opposite side from their origin. They follow the end of the organism for a short distance before branching off anterolaterally. The posterior lateral flagella arise from the same blepharoplast but extend posteriorly along the notch of the sucking disc. They emerge from the tapering side of the trophozoite 2 or 3 μ from the posterior tip. The ventral flagella arise from the axonemes at the level of the posterior border of the nuclei.

TABLE 1—Length and Width Measurements of Trophozoites of Giardia from Chinchilla, Mouse, Dog, Man, Rabbit, and Guinea Pig

Host	Length (μ)			Width (μ)		
	maximum	minimum	mean	maximum	minimum	mean
Chinchilla [1]	17.01	11.34	15.37	12.15	8.10	9.24
Dog	17.01	11.34	14.04	10.53	7.29	8.74
Mouse	13.77	8.90	11.40	10.53	7.29	8.50
Man [2]	20.2	9.2	13.7	10.2	5.0	7.5
Guinea pig	12.0	8.0	10.7	10.1	5.6	7.2
Rabbit	18.7	12.9	15.8	11.0	7.7	9.1

[1] In the chinchilla, dog, and mouse, measurements were taken of at least 50 trophozoites in each instance.

[2] Measurements of trophozoites from man, guinea pig, and rabbit are taken from Hegner.[12] It should be noted that the fixation method used by Hegner was slightly different from the method used during these studies. However, where comparable, Hegner's measurements were very similar to those given here.

Taken in part from a thesis submitted in partial fulfillment of the requirements for the degree of master of science in veterinary medicine at Alabama Polytechnic Institute, Auburn.

The author expresses his appreciation to Jess and C. A. Cutler, Inc., for the research fellowship which made this study possible.

The author also acknowledges the untiring assistance of Dr. W. S. Bailey, Department of Pathology, Alabama Polytechnic Institute, and Dr. P. W. Newberne of the Evergreen Chinchilla Ranch, Columbus, Ga.

A large, flat, sucking disc with a V-shaped posterior border is located on the ventral side of the trophozoite. This disc averages about 9 μ in length and 12 μ in width. On either side of the axonemes, and just posterior to the anterior blepharoplasts, are located two spherical nuclei approximately 3 μ in diameter. Just posterior to the sucking disc, there are two dark-staining bodies approximately 5 μ in length which in many instances appear to be fused together. They lie perpendicular to the axonemes and usually appear to point slightly ventrolaterally. These structures have formerly been known as parabasal bodies but recently Felice [12] has suggested the name "median bodies" as being more appropriate.

The trophozoite stage of the Giardia species found naturally in the chinchilla was uniform in all animals with respect to the over-all size of the organism and the arrangement of the internal structures. The trophozoite length and width of the Giardia spp. found in the chinchilla is compared to those found in 5 other animals in table 1. An examination of these measurements reveals the trophozoite of the chinchilla to be one of the largest yet studied, both in length and width. A marked similarity in size is evident between the Giardia spp. of the chinchilla and *Giardia duodenalis* of the rabbit.

Two types of Giardia cysts were observed in some of the chinchillas studied. One type was ovoid and averaged about 12 μ in length by 8 μ in width. The other type was practically spherical and averaged about 9 μ in diameter. Most of the cysts observed had four nuclei concentrated near the anterior end. From each nucleus a dark-staining axoneme extends backward into the posterior end of the cyst. The paired median bodies were present in all cysts, but their location within the cysts varied greatly. In many of the cysts, remains of the flagella were clearly visible. Due to the variation in the size of the Giardia cysts between individual chinchillas, little value can be placed on cyst dimensions as a means of species differentiation.

LOCATION

Observations were made on the location of the different stages of Giardia organisms in the intestinal tract of each of 3 chinchillas. The results of these observations are shown in table 2, and indicate that the greatest concentration of trophozoites occurs in the duodenum and the anterior jejunum. Most cysts were found in the lower regions of the small intestine and in the cecum. (The total length of the small intestine of the chinchilla is 48 to 50 in.).

HOST SPECIFICITY

Limited experiments were conducted during these studies in an effort to transfer species of Giardia from 1 host animal to another. A brief explanation of these experiments and the results obtained follows.

Chinchilla 20818.—Following negative fecal examinations for eighteen consecutive days this animal received, over a ten-day period, feed heavily contaminated with Giardia cysts from the house mouse *(Mus musculus)*. Daily fecal examinations were continued for sixty-three days, at which time the chinchilla was destroyed. No stages of Giardia were found at any time by the fecal examinations and none was found in the intestinal tract at the time of autopsy.

Two attempts were made to infect white mice with Giardia organisms of the chinchilla. Three, 9-week-old white mice from a stock colony were each given oral doses of 100,000 Giardia cysts of chinchilla origin. One mouse died of shock and injury immediately following dosing. This mouse was opened and direct smears made from material at the anterior portion of the duodenum. Several freshly excysted trophozoites were observed along with some still intact Giardia cysts. The feces of the remaining 2 mice were negative for all stages of Giardia for the next fourteen days, at which time they were destroyed. No stages of Giardia were found by a careful examination of the complete intestinal tract.

TABLE 2—Location of Giardia in the Intestine of the Chinchilla

Chinchilla No.	Inches from pyloric opening												Cecum	Large intes.
	2	4	6	8	10	12	18	24	30	36	42	48		
1	48T	258T	63T	88T	78T	76T	131T	49T	24T	5T	3T	8T	2T	
							3C	21C	8C	18C	20C	3C	21C	4C
2	0T	7T	9T	20T	21T	60T	37T	23T	9T	1T	2T	9T		
								1C	3C	7C	8C	10C	15C	1C
3	45T	59T	45T	52T	44T	40T	21T	21T	18T	2T	1T			
								2C	5C	7C	5C	3C	23C	3C

T—Trophozoites in 20 random high power fields; C—Cysts in 20 random high power fields.

In a second experiment, each of 7, 6-week-old mice was given 225,000 cysts of chinchilla origin. Three of these mice were killed seven days after dosing and the remaining 4 mice were killed fourteen days after dosing. Nothing resembling either stage of Giardia was found in any of these mice.

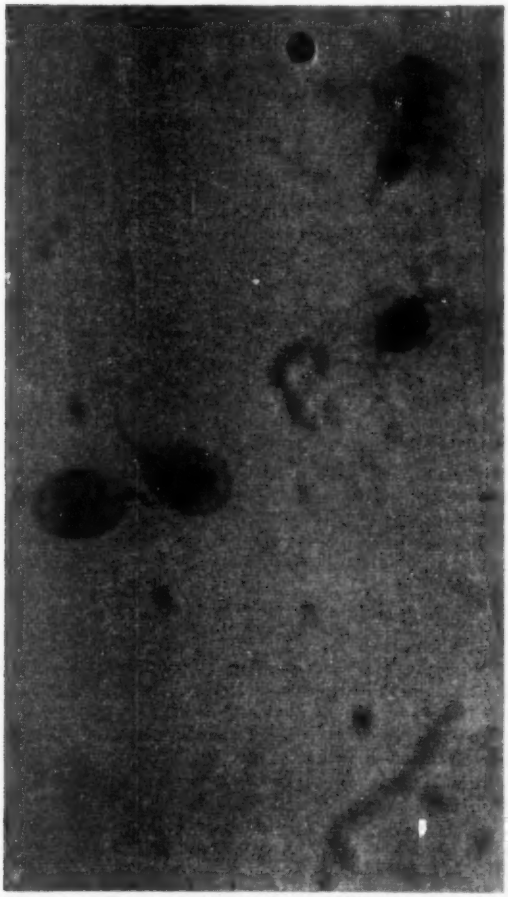

Fig. 1 — Trophozoites of the Giardia species found in the chinchilla. x 450.

Chinchilla 21262.—On March 7, this animal was given 300,000 cysts of *Giardia canis* orally. This dosage was followed by 800,000 cysts of dog origin on March 2. Daily fecal examinations made until April 27 failed to show any evidence that *G. canis* is capable of development or encystment in the chinchilla. Good evidence to show that this chinchilla had no immunity to Giardia was obtained when it began passing moderate numbers of Giardia cysts just six days after receiving 280,000 cysts of chinchilla origin.

DISCUSSION

A number of questions have confronted those who have been concerned with the problem of giardiasis in the chinchilla. What is the origin of the Giardia species found in the chinchilla? Is this organism host-specific? Could mice or rats be reservoir hosts for the parasites and spread them among chinchillas?

By morphological characteristics alone, it appears difficult to differentiate the Giardia organisms of the chinchilla from those found in certain other animals. However, there are very clear-cut morphological differences, both in size and shape, between *Giardia muris* and the Giardia spp. found in the chinchilla.

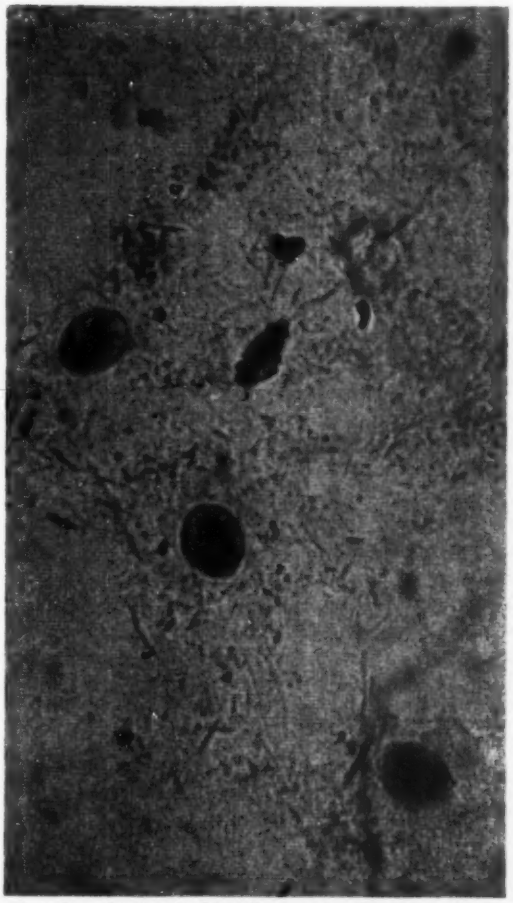

Fig. 2 — Stained Giardia cysts from a direct smear of chinchilla feces. x 450.

Unsuccessful attempts to transfer the Giardia organisms of the chinchilla to the mouse were made during these studies as well as by Morgan.[8] One attempt to transfer *G. muris* to the chinchilla also failed. Although the evidence is not conclusive, it appears very doubtful that the wild mouse is either a native or reservoir host for the Giardia spp. of the chinchilla. Apparently, the only method by which wild mice may spread giardiasis in the chinchilla ward is by mechanical transfer of viable cysts.

By morphological means one could hardly distinguish the Giardia organisms of the chinchilla from *G. duodenalis, Giardia lamblia,* or even *G. canis.* However, cross-transmission experiments made during these studies, and by previous workers, strongly indicate that the members of the genus Giardia are relatively host-specific. Yet the very slight morphological variations between the species of Giardia of some animals hardly supports the theory that each are necessarily different species. Recently, Felice [12] has suggested that the genus Giardia be divided into three groups, mostly on the basis of variations in the shape and location of the median or parabasal bodies. To each of these groups he assigned a species name, *i.e., Giardia agilis, G. muris,* and *G. duodenalis.* To Giardia organisms of each of these groups which are morphologically indistinguishable, yet are found in different hosts and appear to be host-specific, he gives the status of race. Using this method of nomenclature and classification, he proposed the name *Giardia duodenalis* race *chinchillae,* Morgan, 1949, for the Giardia of the chinchilla.

Although this system of classifying the members of the genus Giardia seems to be an appropriate and logical one for the present, it is likely that many changes and revisions will be indicated as more information concerning the physiology, host specificity, and bionomics of these flagellates is obtained.

SUMMARY

Detailed morphological studies were made of the Giardia organisms in the chinchilla in an attempt to determine its relation to Giardia spp. of other animals. The trophozoite of the chinchilla showed a marked uniformity in size with an average length of 15.37 and width of 9.24 μ. A decided morphological difference between the trophozoite of the chinchilla and that of *Giardia muris* was noted.

The greatest concentration of Giardia trophozoites found in the chinchilla were in the first 24 in. of the small intestine. Most of the cysts were concentrated in the cecum.

Attempts to infect chinchillas with *Giardia canis* of the dog and *Giardia muris* of the mouse were unsuccessful. Giardia of the chinchilla did not survive in white mice, although excystation did occur following the oral administration of viable cysts.

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Giardiasis in the Chinchilla. II. Incidence of the Disease and Results of Experimental Infections

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Literature Cited

McIntyre[1] stated that Giardia organisms in the chinchilla were first observed in the laboratory of the Los Angeles County Livestock Sanitary Division in 1943. Morgan[2] reviewed a case report of a heavy Giardia infection in a chinchilla in Wisconsin. Goldsby[3] reported having diagnosed several cases of giardiasis at autopsy in North Dakota. There is little reliable information on the incidence of giardiasis in various animals. The incidence of giardiasis in man reported by Faust and Headlee (15.6 %)[4] agrees very closely with that demonstrated by Cateott (17.7 %)[5] and Choquette (14.7 %)[6] in dogs.

The pathogenicity of Giardia spp. in both man and animals has long been a controversial subject. On the basis of clinical observations, Choquette surmised that most cases of giardiasis in dogs occur in young animals and that the condition is often secondary to an infectious disease such as distemper. Veghelyi[7] reported observations made on 155 children with giardiasis. Most of the children showed a greatly impeded growth development and, usually, anemia. He suggested that the pathogenicity of Giardia organisms is due mainly to mechanical conditions, the absorptive surface of the intestine being partially paralyzed due to layers of the parasites covering the epithelial surface. This mechanical obstruction probably retards all absorption from the intestine but seems to be more specific for fats. Veghelyi found that heavily infected children often eliminate as much as 80 per cent of the consumed fats in the feces. (In a normal child, not more than 8 to 10 per cent of the total fat intake is eliminated unchanged.) Katsampes *et al.*[8] confirmed Veghelyi's work regarding fat absorption by use of the vitamin A absorption test for fats. No reports are available regarding any studies made to determine the possible pathogenicity of Giardia spp. in the chinchilla.

Methods

The materials and methods used were the same as those described for part I (*see* pp. 71, this issue).

Taken in part from a thesis submitted in partial fulfillment of the requirements for the degree of master of science in veterinary medicine at Alabama Polytechnic Institute, Auburn.

The author expresses his appreciation to Jess and C. A. Cutler, Inc., for the research fellowship which made this study possible.

The author also acknowledges the untiring assistance of Dr. W. S. Bailey, Department of Pathology, Alabama Polytechnic Institute, and Dr. P. W. Newberne of the Evergreen Chinchilla Ranch, Columbus, Ga.

Incidence of Infection

At the beginning of this study, an attempt was made to determine the incidence of Giardia infections at the Evergreen Chinchilla Ranch. Several factors made an accurate survey rather difficult. Because the entire ranch was maintained for breeding purposes, all animals were caged in pairs; and a representative group of animals could not be individually isolated. Therefore, this survey was made on the cage rather than the animal basis.

Fecal samples were collected from each cage of ward A once each week for four weeks. A total of 22 cages (20.95 %) of those examined was found to house chinchillas positive for giardiasis.

Experimental Infections

Eight chinchillas were infected with varied doses of Giardia cysts and the course of the infection was studied for forty-five days of the patent period or until the death of the animal. Daily fecal examinations were made by both the direct smear and concentration methods. Five of these chinchillas were housed and fed in the routine manner of the commercial ranch. The others were subjected to poor sanitary surroundings for the purpose of studying the effect produced on the Giardia infections. With the exception of chinchilla 24692, all animals were found negative for giardiasis on 30 or more consecutive daily examinations just prior to dosing with Giardia cysts.

The manner by which the chinchillas were infected, the prepatent period, and a very brief account of the results obtained are shown in table 1. Four of the 8 chinchillas showed no signs or symptoms of disease. There seemed to be little or no correlation between the size of the infective dose and the development of clinical symptoms of disease. Three of the 4 chinchillas which failed to show symptoms continued to discharge moderate numbers of Giardia cysts as long as forty-five days after the beginning of the patent period.

Symptoms and Lesions Developed

Three of the 4 animals which showed signs died. In every instance, the first abnormality noticed was soft, discolored, amorphic droppings which occurred simultaneously with large numbers of Giardia cysts. In 2 animals, a marked diarrhea

developed. The Giardia cyst count during these periods often became extremely high. Chinchilla 10903 eliminated an average of 2,000,000 cysts per gram of feces over a period of fifteen days.

Chinchillas 17965 and 21262 showed the diarrheic symptoms for only a short time, after which they developed almost complete anorexia with a resulting cessation of all fecal eliminations. Some evidence of impaction was noted in both animals prior to death which occurred from three to seven days after the diarrhea ceased. Gross lesions were essentially the same in both animals. Large numbers of trophozoites were found in the small intestine and many cysts in the cecums. Many small, raised, white necrotic foci were present in the wall of the cecum and large colon (fig. 1). A marked degree of fatty change was evident in the liver, and a mild hemorrhagic gastritis was also noted. Apparently pure cultures of bacteria of the genus Pseudomonas were isolated from the liver, heart, and kidney of both chinchillas.

Chinchilla 10903 presented a condition somewhat different. No anorexia was evident at any time and no impaction developed. The chinchilla became very emaciated before death. Autopsy examination revealed large numbers of Giardia organisms as well as a heavy infection of *Hymenolepis nana* var. *fraterna*. (One of the Giardia cyst-donor animals was mildly infected with Hymenolepis and, in this instance, a few tapeworm eggs were included with the original Giardia cyst dosage because of the difficulty involved in removing them.) A more detailed report of the tapeworm infection is given by Shelton and Bailey.[9] Two large circumscribed abscesses were noted in the liver and the thoracic cavity was filled with a sanguineus exudate. Aside from emaciation there were no other gross lesions. No Pseudomonas organisms were isolated from the heart, liver, and lungs.

Effects of Unsanitary Surroundings

Three chinchillas (24692, 21262, and 101) were kept in small, solid-bottomed cages where they had access to their own feces. Although these cages were cleaned daily, the feet and hair could readily become contaminated with Giardia cysts. This was especially true after the droppings became soft or diarrheic. Two of these chinchillas developed heavy cyst discharges and diarrhea soon after the original infection, while the third animal (24692) apparently became free of the parasites about twenty days after the first cysts were found.

Chinchilla 101 was removed from the unsanitary cage at the time the cyst discharge reached a daily average of 1,500,000 cysts per gram of feces. The animal was moved to a clean wire-bottom cage each day for three consecutive days. Special measures were taken to prevent the

TABLE 1—Results of Experimental Infection of Susceptible Chinchillas with Viable Giardia Cysts

Animal No.	Cysts administered (No.)	Prepatent period (days)	Results
24692	Given feed contaminated with feces containing many Giardia cysts	11	Slight infection noted over a period of 20 days. Autopsy after 63 days neg. for giardiasis.
23661	1 dose, 500,000 cysts	8	Mild infection, asymptomatic, positive for 45-day period.
21156	1 dose, 820,000	6	Mild infection, asymptomatic, positive for 45-day period.
102	1 dose, 700,000 2 days later 1 dose, 200,000	8	Moderate infection, asymptomatic, positive for 45-day period.
101	1 dose, 300,000	6	Soft, discolored, amorphic droppings simultaneous with heavy cyst discharge. Positive after 45 days.
17965	1 dose, 810,000	8	Anorexia and cyst discharge with soft droppings 14th day; death 16th day. Pseudomonas spp. isolated.
10903	1 dose, 300,000 1 dose, 320,000 1 dose, 220,000 Given over period of 15 days	8	Soft, discolored, amorphic droppings simultaneous with extreme cyst discharge beginning 30th day. Marked loss of weight noted; death on 45th day. Negative for Pseudomonas organisms.
21262	1 dose, 280,000	6	Soft, discolored feces which soon became watery diarrhea. Diarrhea accompanied by heavy cyst discharge. Noted by 8th day. Anorexia apparent after 11th day; death 18th day. Pseudomonas spp. isolated.

soft feces from adhering to the cage floor. Within three days the consistency of the feces returned to normal and remained so through the remainder of the 45-day period of observation. However, the animal continued to discharge moderate numbers of cysts at five- to nine-day intervals.

PREPATENT PERIOD AND ENCYSTMENT CYCLES

The prepatent period of giardiasis in the 8 experimentally infected chinchillas ranged from six to eleven days.

Studies made of 8 chinchillas infected with Giardia indicate that the encystment period is subject to wide and sharp fluctuations. The encystment periods noted in the experimentally infected animals were very similar to those observed in naturally infected chinchillas. Although a definite cycle was evident in most chinchillas, it was quite variable and inconsistent. However, the interval between the maximum cyst output was usually from five to nine days. Usually the duration of cyst output ranged from one to three days. During the next several days no cysts would be eliminated. An exception to this generalization may be noted in instances of extremely heavy infections when large numbers of cysts may be eliminated daily for several days or weeks in succession. Trophozoites were never found free in chinchilla feces during this study, even under conditions of heavy infection and complete diarrhea. On two occasions, trophozoites were noted in mucous strings from diarrheic chinchilla feces.

DISCUSSION

Several workers have observed Giardia infections in chinchillas representing widely separated geographical areas of the United States. However, there is no information available concerning the incidence of infection from any of these locations. In the limited survey made during this study, approximately 21 per cent of the cages in one ward of a large commercial ranch were found to house infected animals. Additional studies must be made in other areas before any conclusions can be reached concerning the true incidence of this infection. It is interesting to note that the incidence of infection demonstrated in this survey is very similar to that in dogs as determined by Catcott (17.7 %) and Choquette (14.7 %).

From clinical observations of giardiasis in the chinchilla, the fact is readily established that some chinchillas infected with this parasite show intestinal disturbances which seem to be produced by the parasite, but others appear to have asymptomatic infections. Probably, symptoms develop in chinchillas only when heavy Giardia populations are present. Animals infected to this extent are subject to digestive disorders due to the mechanical effect produced by millions of Giardia trophozoites adhering to the mucosal surface. The factor or factors responsible for the intensity of Giardia infections are not definitely known. We may surmise repeated reinfections and complicating bacterial or intercurrent parasitic infections to be predisposing factors in many instances. The possibility that acquired resistance on the part of the host may limit the infection in some chinchillas should be considered.

Fig. 1—Exposed abdominal cavity of chinchilla 21262 showing white necrotic foci in the wall of the cecum and large colon.

The results of the experimental infections made during this study tend to support this hypothesis. Of the 8 animals experimentally infected, 3 died by the end of the 45-day period of observation. Each of these animals showed a change in the color and consistency of the fecal eliminations a few days prior to death. The lesions found on autopsy of 2 of these chinchillas were almost identical with those found by Newberne [10] during a natural disease outbreak. Pure cultures of Pseudomonas were isolated from the internal organs of each of these animals.

The gradual weight loss and the long period of cyst discharge noted in chinchilla 10903 presented a different anamnesis than any other noted in this study. No bacteria of the genus Pseudomonas, or any other bacteria known to be pathogenic to chinchillas, were isolated from the organs of this animal. Apparently, death in this instance was due to the combined effects of Giardia and Hymenolepis infections.

Although the number of animals used in this experiment was too small for the results to be conclusive, it appears that unsanitary surroundings are instrumental in developing heavy Giardia populations in susceptible chinchillas. Giardia organisms are capable of developing asexually in the small intestine. Yet the rapid rise of Giardia populations seems to be in direct proportion to the amount of exposure to feces containing large numbers of cysts. Whether the Giardia population increase is due to the reingestion of viable cysts, or to other factors associated with unsanitary surroundings can not be ascertained at the present time. It is worthy of note that chinchillas may come in contact with their own feces even when housed under the best conditions unless care is exercised by the caretaker. When the droppings become soft and amorphic they adhere to the wire mesh and may soon form a false bottom over part of the cage floor. If immediate steps are not taken to correct this condition, reinfection is practically inevitable.

SUMMARY

A survey for incidence of Giardia infection at the Evergreen Chinchilla Ranch revealed that 20.95 per cent of the cages housed infected animals.

Eight Giardia-free chinchillas were given oral doses ranging from 280,000 to 900,000 Giardia cysts. Giardiasis was established in all animals, but in 1 chinchilla the infection was apparently self-limiting. Three of the 8 animals died on or before the end of forty-five days. All 3 animals developed heavy Giardia infections accompanied by abnormal fecal eliminations a few days prior to death. Descriptions of the symptoms and necropsy findings are given.

Limited observations from this study suggested that reinfections from contact with contaminated feces may account for the extreme Giardia populations sometimes developed.

The prepatent periods for Giardia in the chinchilla ranged from six to eleven days and the encystment cycle was found subject to wide fluctuations. The interval between maximum cyst output usually varied between five and nine days unless the infection was extremely heavy.

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The Blood Picture of Muskrats under Pentobarbital Sodium

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THE MUSKRAT, *Ondatra zebethica zebethica* (Linnaeus), has been studied chiefly by two major groups, conservationists and parasitologists. Conservation workers have reported its feeding habits, reproductive cycle, and other ecological data related to its survival in nature. Parasitologists have found this rodent a fertile source of parasites for taxonomic studies. Most of the available literature on this marsh dweller is concerned with one of these aspects.

The need for additional data on the muskrat became evident following the appearance of a disease (Errington's disease) which occurred in Iowa in the form of an epizoötic in 1943,[1] and which appeared in Wisconsin in 1946.[2] The usefulness of normal blood values as an aid to the diagnosis of diseases affecting man and animals has long been recognized. Their application in the study of wildlife diseases is necessary because clinical manifestations often are suppressed by the intractability of trapped wild animals.

During the course of a project inaugurated in 1951 to study the nature and causation of Errington's disease, data were collected on the blood picture of 71 muskrats. None of the animals showed evidence of disease at the time blood samples were taken.

METHODS

Standard procedures were used on each muskrat studied so far as it was possible. An attempt was made to obtain a sample of blood from each animal within seventy-two hours of its arrival at the laboratory. All samples were withdrawn between 1:00 p.m. and 4:00 p.m. Because of the difficulty in handling muskrats, pentobarbital sodium was administered intraperitoneally at the rate of 1 ml. per animal. The lack of available superficial veins made intracardial puncture the method of choice in obtaining blood. Approximately fifteen minutes were allowed to elapse after anesthesia before intracardial puncture was made. Five milliliter of blood were withdrawn from each animal in order to make the following determinations: erythrocyte count, leukocyte count, differential leukocyte count, hemoglobin level, and blood cell volume. The blood cell volume was read following centrifugation at 2,500 r.p.m. for one hour. The radius of the centrifuge arm was 15 cm. Hemoglobin determinations were made with a Fisher electrohemometer. Red and white cell counts were made in duplicate using a Spencer bright-line hemacytometer. The differential white cell count was based on the identification of 200 cells in smears prepared from fresh blood and stained with Wright's stain. The results of these studies are presented in table 1. A column has been provided for unclassified cells. These latter cells represent leukocytes which no longer were identifiable.

RESULTS

There was a wide range between the maximum and minimum levels of many of the determinations listed. These findings could not be explained on the basis of infection since those animals showing the greatest variation from the arithmetic means did not all reveal significant tissue changes upon autopsy. Such variations could have been entirely physiological in origin but also could have been misinterpreted as pathological variations in the absence of an adequate range based upon the examination of large numbers of healthy specimens.

Excitement will markedly change the normal blood picture of most animals. For this reason, fifteen minutes were allowed to elapse before samples were withdrawn. Comparisons were made possible by standardizing the time interval between anaesthesia and cardiac puncture. Resistance to the handling preparatory to the administration of the anesthetic varied in individual animals. Such differences in behavior strengthened expectation of variation in individual determinations.

MORPHOLOGY OF BLOOD CELL TYPES

Blood smears were prepared with Wright's stain for differential counts and for the morphological studies; a buffer of pH 6.6 was used. Twenty cells of each type

From the Department of Veterinary Science, University of Wisconsin, Paper NS 144. Published with the approval of the director of the Wisconsin Agricultural Experiment Station and supported in part by Pittman-Robertson Project (15-R) of the Wisconsin Conservation Department.

Appreciation is expressed to the game division staff of the Wisconsin Conservation Department who through their own efforts and contacts with fur farmers procured animals for study.

TABLE 1—Blood Picture of 71 Wisconsin Muskrats

	Erythrocytes (10^6 / cmm.)	Leukocytes (10^3 / cmm.)	Packed volume of erythrocytes (%)	Hemoglobin (Gm. / 100 ml.)	m.c.h.	m.c.v.	m.c.h.c.
Animals	71	71	71	71	70	70	70
Range	4.3-8.0	3.3-25.0	34.0-68.0	6.6-19.8	13.4-29.2	64.7-119.0	19.4-38.3
Arithmetic mean and s.e. *	6.38±0.096	7.51±0.349	50.29±0.738	13.65±0.333	21.61±0.476	79.92±1.391	27.16±0.808
Standard deviation	0.805	2.947	6.21	2.81	3.99	11.64	6.76
Coefficient of variation	12.6	39.2	12.4	20.6	18.5	14.6	24.9

	Leukocytes (%)					
	Neutrophils	Lymphocytes	Monocytes	Eosinophils		Unclassified cells
Animals	71	71	71	71**		71
Range	33.0-93.5	5.0-46.0	0.0-10.0	0.0-2.5		0.0-15.0
Arithmetic mean and s.e. *	70.09±1.562	24.92±1.513	2.84±0.225	0.615		1.66
Standard deviation	13.124	12.705	1.916	.....	.....	
Coefficient of variation	18.8	50.9	45.1	125.4	.....	

* Standard error; ** One aberrant measurement (14.5) omitted; m.c.h. — mean corpuscular hemoglobin; m.c.v. — mean corpuscular volume; m.c.h.c. — hemoglobin concentration.

were measured with an ocular micrometer in order to obtain the average size of the individual cells.

The Erythrocyte.—With Wright's stain the erythrocyte was found to be a pale orange-colored biconcave disc averaging 6.79 μ in diameter. Many morphological features and staining reactions of this cell were observed which in other mammalian species have been interpreted as indicative of certain forms of blood dyscrasia.

Polychromatophilia was a frequent finding. The erythrocytes involved were pale blue in color and were present in fairly large numbers. Howell-Jolly bodies were observed in most smears examined. The finding of numerous normoblasts in the same smears strongly supported the idea that they were particles of erythrocyte, nuclear debris and not a previously undescribed hemosporidian.

Target cells were frequently encountered in the smears examined. These cells were within the normal size range, but their centers and peripheries were pale pink in color. The cells showed an achromatic midzonal area.

The basophilic normoblast was found to be a small cell averaging 5.46 μ in diameter. It contained a dark, bluish purple nucleus frequently ectopic in position. The chromatin was coarsely granular and the periphery of the nucleus was irregular in outline. Immediately around the nucleous was a halo of pale, pink cytoplasm. The remainder of the cytoplasm was basophilic and no granules were observed. This cell should be distinguished from the small lymphocyte which is similar in morphology.

The Lymphocyte—The lymphocyte of the muskrat was found to average 9.74 μ in diameter. This cell was regular in outline with a dark, bluish purple nucleus and strongly basophilic cytoplasm. The cytoplasmic-nuclear ratio was small. The nuclear chromatin was dense and coarse in appearance. Immature lymphocytes were numerous; many such cells were found which contained nucleoli. Azurophil granules sometimes were observed in the cytoplasm of mature lymphocytes. These granules varied in number from 4 to 12 and were less than 1 μ in diameter.

The Monocyte.—The monocyte was found to be a large cell averaging 16.09 μ in diameter. The cytoplasmic-nuclear ratio was larger than that of the lymphocyte. The

nucelus was irregular in shape and frequently appeared to be folded on itself, creating a lobular appearance. As many as four lobes were observed. The nucleus did not stain as deeply as that of the lymphocyte and its chromatin was less coarse and granular. Nucleoli were observed rarely. The cytoplasm appeared as a homogenous, pale blue material studded with fine, bluish purple granules. These gave a frosted appearance to the cytoplasm. On no occasion did the granules approximate the size of those observed in the lymphocyte.

The Neutrophil.—The neutrophil was the most numerous leukocyte in the differential count. It was found to have a diameter averaging 14.63 μ. The lobulations of the nucleus were often poorly defined. As many as eight distinct lobes were observed in mature cells. Frequently, the nucleus was seen to be folded on itself to form a figure of eight; sometimes it was arranged in a clover-leaf pattern. The nuclear chromatin was coarsely granular and stained dark purple. The cytoplasm contained numerous fine pink granules. The cytoplasmic membrane was poorly defined.

The Eosinophil.—The average size of the eosinophil of the muskrat was found to be 16.81 μ. This granulocyte was peculiar in that the nucleus was rarely segmented. The nucleus appeared as a doughnut-shaped structure, finely granular, and pale, bluish purple in color. This type of nuclear configuration also was observed in bone marrow smears. The granules of the eosinophil measured 1.42 μ in diameter. They filled the space created by the peculiar configuration of the nucleus and were also present in the peripheral cytoplasm.

The Basophil.—No granulocyte could be distinguished as a basophil in the 71 blood smears examined.

SUMMARY

1) A study of the blood picture of 71 apparently healthy Wisconsin muskrats under pentobarbital sodium anesthesia revealed the following average blood values and standard errors: erythrocytes, 6.33 ± 0.096 million per cubic millimeter; leukocytes, 7.51 ± 0.349 thousands per cubic millimeter; packed volume of erythrocytes, 50.29 ± 0.738 per cent; hemoglobin, 13.65 ± 0.333 Gm. per 100 ml.; mean corpuscular hemoglobin, 21.61 ± 0.476 μg.; mean corpuscular volume, 79.92 ± 1.391 c.μ; and mean corpuscular hemoglobin concentration, 27.16 ± 0.808 per cent.

2) The differential leukocyte counts revealed the following mean percentages and standard errors: neutrophils, 70.09 ± 1.562; lymphocytes, 24.92 ± 1.513; monocytes, 2.84 ± 0.225; eosinophils, 0.615; and unclassified cells, 1.66.

3) Failure to find a basophil suggests that such a cell may be lacking in the blood of the muskrat. Kennedy [3] found that foxes lacked eosinophils.

4) The large number of normoblasts observed suggests that the hematopoietic system of the muskrat is very active.

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The Gross Anatomy of the Digestive System of the Mink
I. The Headgut and the Foregut

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THIS STUDY was undertaken with the express purpose of presenting an initial description of the gross anatomy of the digestive system of the North American mink, *Mustela vison*.

A review of the literature proved relatively unproductive of specific references to the anatomy of the digestive system of the North American mink. Wood [1] reported that the stomach averaged 0.7 per cent of the body weight. More information was available on European species. Neseni [2] gave 0.5 Gm. as the weight of the parotid gland of the European polecat, *Mustela putorius*. Oppel [3] indicated that the parotid, mandibular, and major sublingual glands were present and that the minor sublingual gland was absent in this species. He classified the mandibular gland as mucous and the major sublingual gland as mucoserous. Bronn [4] classified the latter as a mucous gland. Bronn also described two pairs of vallate papillae on the tongue of the ermine, *Mustela erminea*.

MATERIALS AND METHODS

The mink used in this study were supplied by the Coöperative Fur Animal Disease Research Laboratory, State College of Washington, and the U. S. Department of Agriculture, Bureau of Animal Industry, at Pullman, Wash. Animals of both sexes, different ages, and varying stages of nutrition were represented. The majority were mink of pelting age—about 6 months. Nineteen individuals used in feeding experiments, which exerted no apparent effect on normal growth rate, were 3 months old. A few were over a year old. Some were embalmed for detailed dissection, but the majority were dissected in the fresh condition.

The tongues were excised by severing the lingual attachments of the extrinsic lingual muscles and the attachment of the root of the tongue from the basihyoid bone. The excised tongues were measured. The teeth were examined in fresh heads and in skulls which had been cleaned and sculptured to expose the roots. Observations starting at 1 week of age were made on two litters of 3 kits each to determine the age of eruption of the deciduous teeth. Observations were continued to determine the age of replacement of the deciduous teeth by the eruption of the permanent teeth. These observations were supplemented by examining the teeth of 28 kits 1 month old or older, which were destroyed in "yellow fat" nutrition experiments. The salivary ducts were dissected with the aid of a dissection microscope having a magnification of 15 diameters. The salivary glands were weighed immediately after their excision. Microscopic examinations of tissue sections were necessary to determine the secretory type of the glands. The cavity of the digestive pharynx was best examined by entering it through a midventral incision.

The total length of the esophagus and the lengths of its cervical, thoracic, and abdominal parts were measured *in situ* with calipers. The volume of the stomach was estimated by ligating the pyloric valve and injecting water into the abdominal part of the esophagus near the cardia of an empty stomach. The length of the lesser curvature was measured by transecting the pyloric valve and manipulating the stomach so that the lesser curvature was brought into a straight line. The stomach was separated from the esophagus, incised, and washed in physiological saline solution; then it was blotted dry and weighed. Forty cubic centimeters of a barium sulphate suspension were introduced into the stomach of a mink (body weight = 625 Gm.) by anesthetizing it with an intraperitoneal injection of sodium pentobarbital and intubating the esophagus with a small metal catheter attached to a syringe. Radiographs were taken twenty minutes later to reveal the position of the stomach and the intestines.

RESULTS

The Oral Cavity.—The oral fissure of the mink is fairly extensive, the labial commissures extending back to a point posterior to the carnassial teeth. The upper lip is far more extensive and mobile than the lower lip. There is a distinct philtrum. Follicles of the tactile hairs extend nearly to the mucocutaneous junction. The orbicularis oris muscle is moderately well developed. A small median frenulum labii superiorus is present. The lower lip exhibits little flexibility. It is fairly loose toward the commissures, but near the lower first premolars it is tightly bound to the gum. At this point, the upper canine tooth extends ventrad superficial to the lower lip. The cheeks can not be considered capacious.

The hard palate is shaped somewhat like an arrowhead. The first two ridges curve

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From a thesis submitted in partial fulfillment of the requirements for the degree of master of science in veterinary medicine, State College of Washington, Pullman. The author is now in the Department of Veterinary Pathology, School of Veterinary Medicine, Oklahoma A. and M. College, Stillwater.

across the entire width of the hard palate. Posterior to these, a median groove separates five transverse ridges on each side. The groove becomes wider and deeper in its posterior part (fig. 4, 1). There is a distinct papilla incisiva and two minute ductus incisivi. In 77 mink ranging from 281 to 425 mm. in body length, the palatal length ranged from 25 to 34 mm. with a mean of 29 ± 2 mm.; the maximum width of the hard palate ranged from 12 to 19 mm. with a mean of 16 ± 1 mm.

The oral mucous membrane is a delicate pink color and presents a smooth surface. Each caruncula sublingualis is leaf-like with a prominent medial projection. A pterygomandibular fold extends from the upper to the lower jaw on each side behind the last molars. The glossopalatine folds lie a short distance posterior to these folds, forming the palatoglossal arch.

The Tongue.—The tongue is thick at the root and becomes thinner and narrower toward the rounded apex. A median groove on the dorsum is deepest over the body, fading out at the apex and the root. Curving rows of well-cornified papillae extend anterolaterad from each side of the groove. These papillae are mainly filiform with a few being fungiform. Four vallate papillae, arranged in a V, are found on the dorsum toward the root of the tongue. Exclusive of the apex, the lateral surface is devoid of papillae. The ventral surface is also smooth. The frenulum linguae is extensive enough to permit good flexibility of the tongue. There is no evidence of a lyssa.

The styloglossus is a broad heavy muscle. It originates not only from the stylohyoid bone but also from the lateral surface of the tympanic bulla. The majority of its fibers enter the middle half of the tongue. The other lingual muscles do not vary appreciably from those in the dog.

The weight of the tongue ranged from 1.0 to 2.2 Gm., with a mean of 1.6 ± 0.3 Gm. in 77 mink ranging from 400 to 1,450 Gm. in body weight. The relative weight was less in larger mink. The length of the tongue ranged from 31 to 45 mm., with a mean of 40 ± 3 mm.

The Teeth.—The formula of the permanent teeth of the mink is:

$$2\left(\mathrm{I}\frac{3}{3}\ \mathrm{C}\frac{1}{1}\ \mathrm{P}\frac{3}{3}\ \mathrm{M}\frac{1}{2}\right) = 34$$

The labiolingual diameter of the incisor teeth exceeds the transverse diameter. The upper first and second incisors are small; the upper third incisor is noticeably larger. The lower first incisor is smaller than the lower second and third incisors which are of about equal size. The lower incisors are irregularly placed, the second incisor occupying a position posterior to the first and third incisors. When the mouth is closed, the lingual surfaces of the upper first and second incisors overlap the labial surfaces of the corresponding lower incisors: the upper and lower third incisors are in contact, the crown of each upper third incisor being deflected laterad. Each incisor tooth has a single root which occupies an alveolus in a premaxilla in the upper arcade or in the mandible in the lower arcade.

The canine teeth are large and possess long roots which curve back into their respective alveoli in the maxilla or mandible. The length of the root of each canine tooth

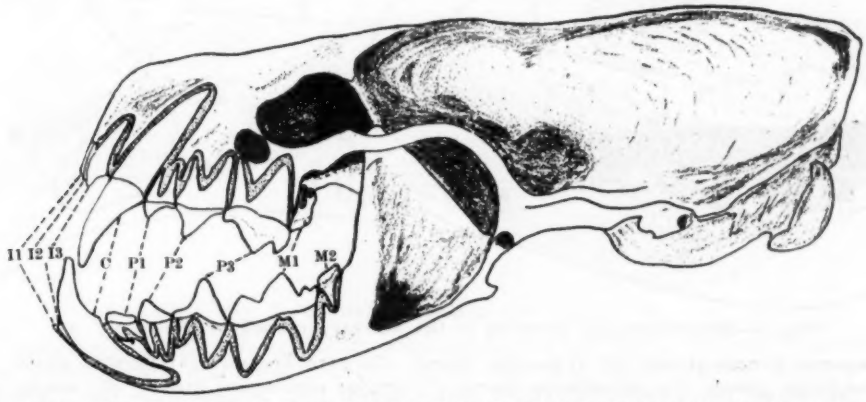


Fig. 1—Skull of an adult mink sculptured to reveal the roots of the teeth. x 1.5.

exceeds the length of the crown. The latter is marked by a groove on its concave surface. The lower canine teeth are somewhat shorter than the upper canine teeth. Each lower canine tooth occupies a position labial to, and between, the upper canine and upper third incisor. The upper canine incisor projects slightly laterad and extends ventrad superficial to the lower lip.

In the lower arcade, the three premolar teeth and the first molar tooth are sectorial, each having a large middle cone, a small anterior cone, and a small posterior cone. The two small cones are greatly reduced on the lower premolars but well developed on the lower first molar. Each lower premolar has two roots which project into alveoli in the mandible. The two roots of the large first molar or carnassial tooth assume more massive proportions. The second molar is a small tooth with a flattened crown and a single root (fig. 1).

The three upper premolars are also sectorial in nature, the first and second resembling the lower premolars. Each of the latter two teeth has two roots which project into alveoli in the maxilla. The upper third premolar has a large middle cone, a smaller posterior cone, and two small anterior cones. The lingual cone is the larger of the two latter cones. Two small roots project from these cones into alveoli in the maxilla, the medial alveolus projecting into the maxillary sinus while the lateral alveolus ends at the rim of the infraorbital foramen. A third, larger root is lodged in an alveolus lateroposterior to the infraorbital foramen. The upper molar tooth is tuberculate. Its long axis is transverse. There is a depression in the middle of the masticatory surface which separates a larger lingual part from a smaller buccal part. This tooth possesses three roots. Two roots which arise from the buccal part are lodged in a common alveolus immediately posterior to the large root of the third premolar. A larger root from the lingual part is lodged in an alveolus with a wall so thin that the apex of the root projects through the bone in a cleaned skull.

When the jaws are closed, the first two upper premolars occupy the spaces between the three lower premolars, the upper teeth slightly overlapping the buccal surface of the lower teeth. The upper third premolar overlaps the lower first molar. The posterior cone of the latter tooth rests in the depression on the masticatory surface of the upper molar.

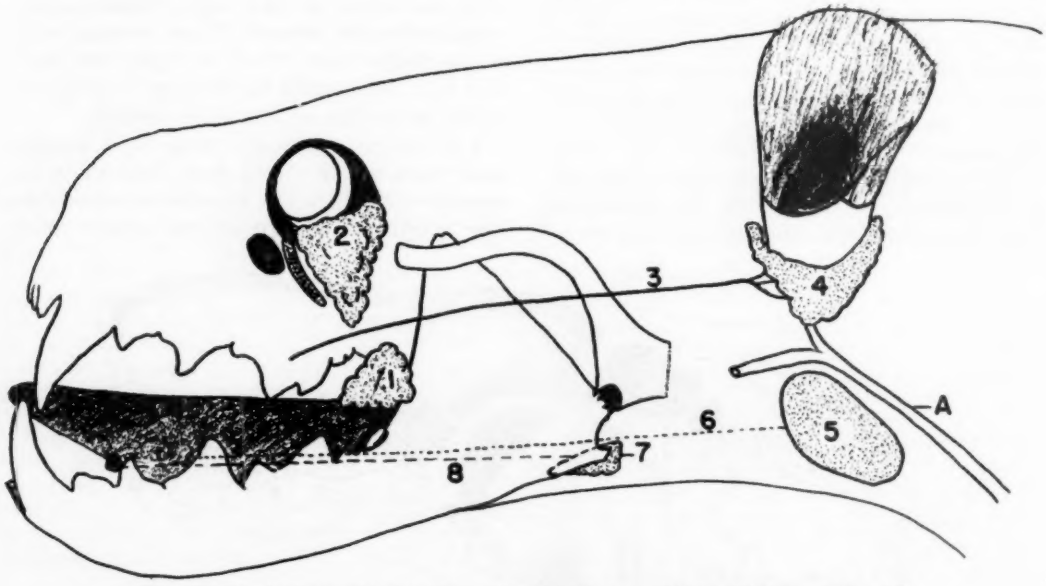


Fig. 2—Semischematic drawing of the salivary glands of the mink. x 2.

1) Ventral buccal gland; (2) zygomatic gland; (3) parotid duct; (4) parotid gland; (5) mandibular gland; (6) mandibular duct; (7) major sublingual gland; (8) major sublingual duct; (A) external jugular vein.

The formula of the deciduous teeth of the mink is:

$$2\left(\text{Di}\frac{1}{0}\ \text{De}\frac{1}{1}\ \text{Dp}\frac{3}{3}\right) = 18$$

Prominent ridges mark the gums of mink kits during the first two weeks of their postnatal existence. The deciduous teeth erupt between 3 and 4 weeks of age. The canine incisors erupt first, then the upper third incisors with the premolars following in order. The permanent first and second incisors erupt between 6 and 7 weeks of age; the permanent third incisors erupt in the eighth week. Eruption of the remaining permanent teeth takes place between 8 and 10 weeks of age. The upper second and lower third premolars and the second molars are the last permanent teeth to erupt.

The deciduous upper second and lower third premolars more nearly resemble the permanent carnassial teeth than the teeth which replace them. Similarly, the deciduous upper third premolar resembles the permanent upper molar. The permanent canine teeth come in anterior to their respective deciduous antecedents. The permanent premolars erupt at the lingual side of the deciduous premolars.

The Salivary Glands.—The mandibular gland is oval with a flattened superficial surface. It is covered by superficial fascia and cervical cutaneous muscle. The deep surface presents a contour which is adapted to the depression between the digastricus and sternocephalicus muscles upon which it lies. The deep surface is also related to the medial retropharyngeal lymph node. Ventrally the gland is related to the sternohyoideus muscle; dorsally, to the external maxillary and external jugular veins. The gland, which is enclosed in a fibrous capsule, is normally buff colored. It is a mixed, predominantly mucous gland. The mandibular duct is formed by the union of several smaller radicles at the central part of the deep surface of the gland. The duct emerges under the anterior border, crosses the superficial surface of the digastricus, and dips under the external maxillary vein. Then it passes around the dorsal border of the digastricus, contacting the masseter muscle as it does so. The mandibular duct proceeds anteroventrad along the dorsal border of the major sublingual gland to a point where it is accompanied by the gland's smaller duct. The lingual nerve crosses the ventrolateral aspect of the two ducts. The ducts

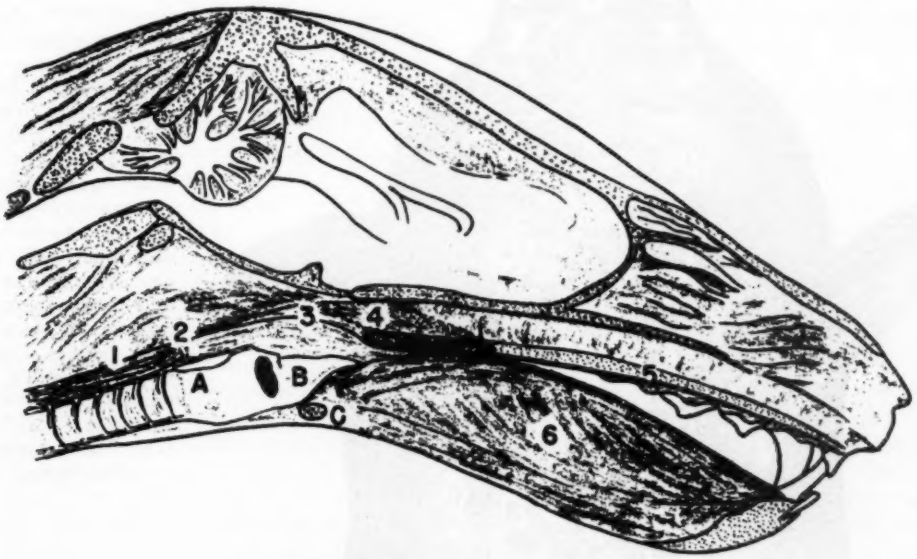


Fig. 3—Sagittal section of the head. x 1.5.

1) Esophagus (slightly dilated); (2) pharyngeosophageal limit; (3) palatopharyngeal fold (forms lateral margin of intrapharyngeal opening); (4) soft palate (slightly contracted); (5) hard palate; (6) tongue; (A) cricoid cartilage; (B) epiglottis; (C) basihyoid bone.

extend forward between the styloglossus and mylohyoideus muscles and then up under the oral mucous membrane to terminate at the caruncula sublingualis. The relative positions of of the salivary glands are illustrated semischematically in figure 2.

The parotid gland is a pale buff, loosely lobulated organ. Its shape is roughly triangular with the dorsal border notched to fit the auricular cartilage. The gland is covered superficially by the cutaneous and parotido-auricularis muscles. The deep surface is related to the internal maxillary vein, the facial nerve, and the base of the ear. The great auricular vein runs along the posterior border. The apex of the gland contacts the mandibular lymph node just above the external maxillary vein. The parotid gland is serous. The parotid duct is formed by two or three major radicles. It leaves the anterior border of the gland and proceeds across the superficial surface of the masseter muscle ventral to the dorsal buccal nerve. The duct is slightly smaller than the nerve. After crossing the masseter, the duct dips under the facial vessels, penetrates the cheek, and opens above the upper third premolar.

The major sublingual gland, the smallest of the salivary glands, is roughly the shape of a right triangle. It is located between the digastricus and mylohyoideus muscles laterally and the styloglossus medially at the level of a transverse plane through the angular process of the mandible. Posteriorly it is related to the external carotid artery. The color of this mixed gland is slightly darker than the mandibular gland. The major sublingual duct leaves the middle of the dorsal border of the gland and assumes a position medial to the mandibular duct which it accompanies to the caruncula sublingualis. The minor sublingual gland is absent.

The zygomatic gland is situated between the ventrolateral surface of the periorbita and the zygomatic arch. Its deep surface is

Fig. 4—Frontal section of the head with the digestive pharynx exposed by a midventral incision. x 1.5.

1) Hard palate; (2) anterior border of the glossopalatine fold; (3) soft palate; (4) palatine tonsil; (5) palatine arch; (6) palatopharyngeal fold; (7) palatopharyngeal arch; (8) intrapharyngeal opening; (9) esophageal vestibule; (10) pharyngoesophageal limit; (11) esophagus; (12) tongue (split longitudinally into halves); (A) cricoid cartilage; (B) epiglottis; (C) basihyoid bone.

related to the pterygoid muscles, the internal maxillary artery and the maxillary nerve, as well as to the periorbita. This lobulated gland is irregularly pyramidal in shape and pale buff in color. Several ducts enter the oral cavity behind the upper molar tooth.

A well-developed ventral buccal gland lies between the orbicularis oris anteriorly and the facial vessels posteriorly. It is slightly paler than the color of muscle; its shape is approximately that of an equilateral triangle. Minute ducts empty into the vestibule just posterior to the labial commissure. Both the zygomatic and ventral buccal glands are mixed, predominantly mucous, glands.

The weights of the salivary glands are summarized in table 1. The 28 mink from which the glands were taken ranged in weight from 422 Gm. to 1,447 Gm. with a mean weight of 729 ± 77 Gm. The minimum, maximum, and mean weights are given for single glands while the mean milligrams per 100 grams of body weight includes the combined weights of the paired glands. A marked increase in milligrams of salivary gland per 100 grams of body weight takes place as age increases from 3 months to pelting age—about 6 months.

The Digestive Pharynx.—The entire length of the digestive pharynx [5,6] lies ventral to the base of the cranium (fig. 3). The longitudinal axis is only slightly curved. The length of the digestive pharynx from the posterior border of the hard palate to the pharyngoesophageal limit is about 2 cm. The oral pharynx constitutes a substantial part of the total length of the digestive pharynx. The intrapharyngeal opening is ellipsoidal with a longer anteroposterior diameter. It is formed by the strongly concave palatine arch anteriorly and the narrow palatopharyngeal folds laterally and dorsally where these folds unite to form a distinct palatopharyngeal arch. An exposed, flap-like palatine tonsil lies on each side of the concavity of the glossopalatine fold. Each tonsil is about 4 or 5 mm. long and light pink. Figure 4 illustrates the digestive pharynx through a midventral incision.

The laryngeal crown, formed by the aryepiglottic folds, the epiglottis and the paired corniculate and cuneiform cartilages, is relatively low. The cavity of the laryngeal pharynx is thus given a shallow appearance.

The esophageal vestibule is well developed and clearly demarcated from the esophagus by the pharyngoesophageal limit. The latter is a definite annular fold of mucous membrane which can not be effaced by stretching. It lies in a transverse plane which passes through the posterior border of the lamina of the cricoid cartilage.

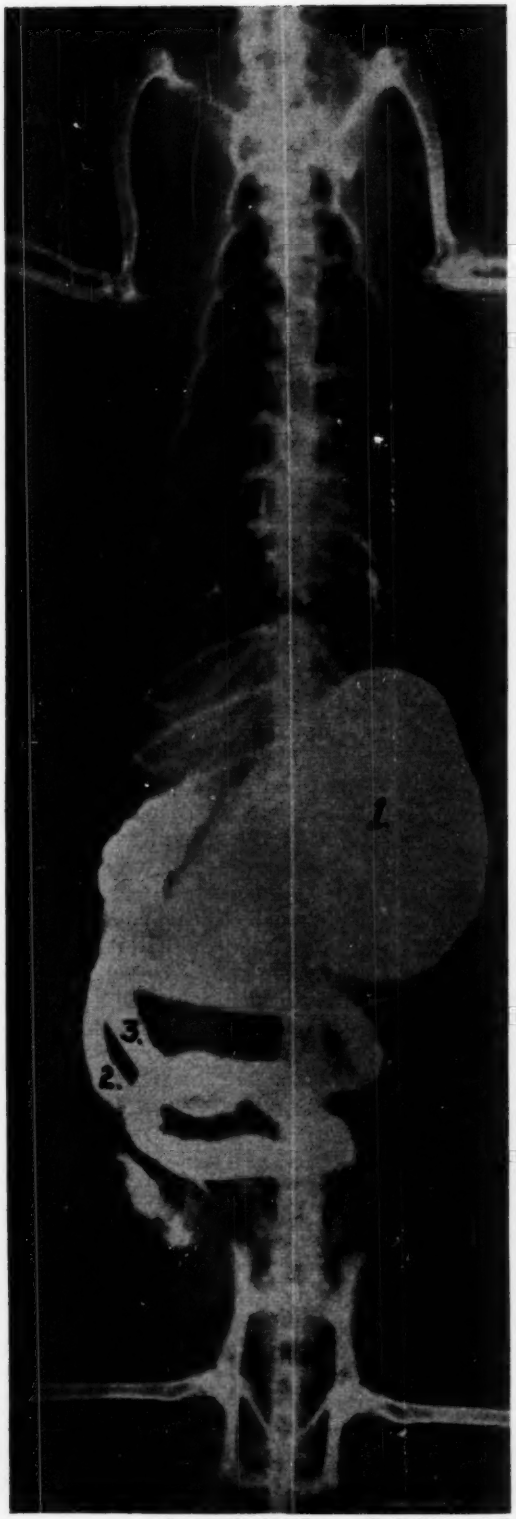
TABLE 1—Weights in Milligrams of the Salivary Glands of 28 Male and Female Mink Over 3 Months of Age

Salivary gland	Range Min. to Max.	Mean weight	Mean mg./ 100 Gm.
Mandibular	160 to 478	332 ± 83*	95
Parotid	52 to 231	114 ± 46	32
Zygomatic	32 to 258	116 ± 52	34
Ventral Buccal	20 to 129	53 ± 21	15
Sublingual	5 to 63	16 ± 10	5

* Standard deviation.

The hyopharyngeus muscle is larger than the other two constrictors of the pharynx. It consists of a chondropharyngeus part and a smaller keratopharyngeus part. The stylopharyngeus muscle is wide. The shorteners of the pharynx are represented by a short muscle mass which arises solely from the pterygoid bone and might properly be called the pterygopharyngeus only. The extrinsic palatine muscles present no differential features.

The Esophagus.—The esophagus begins at the pharyngoesophageal limit and extends in a posterior direction on the trachea, gradually deviating to the left. One third of the way along the neck the cervical part of the esophagus passes deep to the common carotid artery. At the thoracic inlet it lies on the left surface of the trachea. From this point the thoracic part of the esophagus extends to the right, crossing deep to the left subclavian artery and then lying dorsal to the artery. It crosses the right surface of the aorta, passes under the aortic arch, and continues its posterior course ventral to the vessel. Upon reaching the diaphragm the esophagus, accompanied by the dorsal and ventral esophageal nerve trunks, passes through the hiatus esophageus which lies ventral to the fourteenth thoracic vertebra. After passing through the hiatus the relatively long abdominal part contacts the coronary



Legends for figure 5 (left) and figure 6 (right) are on opposite page.

ligament and the left lateral lobe of the liver and ends at the cardia of the stomach. The lumen of the esophagus is wide and dilatable except at its origin and just before passing through the diaphragm, where it is smaller. The mucous membrane is thrown into longitudinal folds.

The esophagus is approximately half the length of the body. In a series of 77 mink, the cervical part of the esophagus averaged 38 per cent, the thoracic part 55 per cent, and the abdominal part 7 per cent of the total length of the esophagus.

The Stomach.—As in most carnivores, the stomach of the mink is subject to a considerable variation in size and position. When the stomach is empty the anterior limit is on a plane perpendicular to the thirteenth thoracic vertebra, and the posterior limit is on a plane perpendicular to the first lumbar vertebra or approximately at the posterior border of the liver. The parietal surface faces toward the liver and the ventral abdominal wall. The visceral surface is related to the intestinal mass and the left limb of the pancreas. The greater curvature faces posteroventrad and to the left. The left half of the greater curvature is related to the gastric surface of the spleen. At this location the gastrosplenic ligament joins the two organs. The lesser curvature faces anterodorsad and to the right. It is intimately related to the papillary process of the caudate lobe of the liver. The lesser omentum extends from the lesser curvature to the portal fissure.

The shape and position of the stomach vary with the degree of distension. The fundus is extensive. As the stomach fills, the fundus moves posteroventrad to the ventral abdominal wall. When the stomach is fully distended it occupies the entire left parachondriac region of the abdominal cavity, pushing the intestinal mass to the right. It expands in a posterior direction, contacting the left kidney and reaching a point about midway between the xiphoid process and the pubic brim. From 40 to 70 cc. of water may be introduced into a stomach before this posterior point is reached. The radiographs in figures 5 and 6 provide the best means of illustrating the shape and position of a mink's stomach following distension with a barium meal suspension. The pyloric antrum is constricted. The pylorus varies the least in position of any part of the stomach; it is located to the right of the midline on a plane perpendicular to the thoracolumbar junction. The pyloric sphincter is moderately well developed. The pyloric opening is directed anterodorsad.

The esophageal mucosa terminates abruptly, forming a thin rim at the cardia. The junction of the region of the cardiac glands with the region of the fundic glands is indistinguishable macroscopically. The deep pink fundic region presents several irregular folds. There are fewer folds in the region of the pyloric glands which is a lighter pink than the fundic region. Enlarged pits with well-defined limits are occasionally seen in the gastric mucosa. They vary in size and occurrence, giving rise to suspicion of a pathological process.

In 77 mink, ranging from 400 to 1,450 Gm. in body weight, the weight of the stomach ranged from 2.5 to 4.8 Gm. with a mean of 3.8 ± 0.8 Gm.; the stomach averaged 519 mg. per 100 Gm. of body weight. The length of the lesser curvature ranged from 65 to 109 mm. with a mean of 85 ± 11 mm.

DISCUSSION

Especial attention is called to the absence of the deciduous incisor teeth exclusive of the deciduous upper third incisors which erupt at about 3 weeks of age. The weight of 0.5 Gm. given for the parotid gland of the European polecat by Neseni [2] was more than twice the maximum weight of the parotid gland of the North American mink. Oppel's [3] observation on the absence of a minor sublingual salivary gland in the European polecat was confirmed in the North American mink. The major sublingual gland of the North American mink was found to be mixed, agreeing with Oppel's classification of the same gland in the European polecat and disagreeing with

Legends for Figures on Opposite Page

Fig. 5—Radiograph of a mink twenty minutes after the introduction of 40 cc. of a barium sulphate suspension into the stomach. Ventral view. x 0.8.

1) Stomach; (2) duodenum; (3) initial part of jejunum.

Fig. 6—Radiograph of the same mink as in figure 5. Lateral view. x 0.8.

1) Stomach; (2) duodenum; (3) initial part of jejunum.

Bronn's [4] description of a mucous gland. The digestive pharynx of the North American mink has a well-developed esophageal vestibule demarcated from the esophagus by a clearly-defined pharyngoesophageal limit. The stomach averaged 519 mg. per 100 Gm. of body weight or 0.52 per cent of the body weight. This is slightly less than the 0.7 per cent of the body weight given by Wood.[1]

It is recognized that the samples of the mink population examined in this study were insufficient to demonstrate the many variations which might be encountered. Before the range of the eruption dates of the teeth can be ascertained many mink will have to be examined. The availability of mink raised in captivity and the need for data on greater numbers of individuals should serve to stimulate further inquiry into the anatomy of the digestive system as well as other systems of the mink's body.

SUMMARY

1) A description of the headgut and foregut of the mink was presented.

2) The hard palate is shaped somewhat like an arrowhead. It is traversed by two complete ridges and five which are separated by a median groove. The mean palatal length is 29 mm.; the mean maximum width, 16 mm. There is a distinct incisive papilla.

3) The tongue has a dorsal median groove and four vallate papillae. The mean weight of the tongue is 1.6 Gm.; the mean length, 40 mm. The lyssa is absent. There are two leaf-like sublingual caruncles.

4) The permanent dental formula is $2\left(I\frac{3}{3}\ C\frac{1}{1}\ P\frac{3}{3}\ M\frac{1}{2}\right) = 34$. The deciduous dental formula is $2\left(Di\frac{1}{0}\ Dc\frac{1}{1}\ Dp\frac{3}{3}\right) = 18$. Eruption of the deciduous teeth takes place between 3 and 4 weeks of age. At 6 weeks the permanent teeth begin to erupt, and by 10 weeks the eruption of the teeth is complete.

5) The five salivary glands in the order of their mean weights are: the mandibular (332 mg.), the zygomatic (116 mg.), the parotid (114 mg.), the ventral buccal (53 mg.), and the major sublingual (16 mg.). The mandibular, zygomatic, and ventral buccal glands are mixed, predominantly mucous, glands. The parotid gland is serous and the major sublingual gland is mixed. The minor sublingual gland is absent.

6) The entire, slightly-curved length of the digestive pharynx lies ventral to the base of the cranium. The palatine tonsils are exposed and flaplike. There is a distinct pharyngoesophageal limit.

7) The esophagus is approximately one half the length of the body. The cervical part is 38 per cent, the thoracic part is 55 per cent, and the abdominal part 7 per cent of the total length.

8) When the stomach is fully distended, it contains from 40 to 70 cc. of ingesta and occupies the entire left parachondriac region of the abdominal cavity. The mean length of the lesser curvature is 85 mm. The stomach's mean weight is 3.8 Gm. or about 0.5 per cent of the body weight.

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The Gross Anatomy of the Digestive System of the Mink
II. The Midgut and the Hindgut

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A lone reference to the anatomy of the midgut of the North American mink *(Mustela vison)* was given by Wood.[1] In over 70 mink, the liver averaged 5.1 per cent of the body weight.

Neseni[2] examined 26 European mink *(Mustela lutreola)*. He reported the absence of a cecum, a familial characteristic. The intestinal canal ranged from 121 to 205 cm. in length with an average length of 160 cm. The ratio of the length of the intestine to body length ranged from 3.4:1 to 6.0:1 with an average ratio of 4.6:1. In presenting data on 2 European polecats *(Mustela putorius)*, Neseni gave intestinal lengths of 160 cm. and 115 cm. with respective ratios to body length of 6.0:1 to 5.1:1. The gall bladder of each contained about 0.5 cc. of bile. The common bile duct opened 1.5 cm. from the pylorus in one examination.

The following data were given in the "Tabulae Biologicae."[3] According to Schutze,[4] the liver constituted 4.6 per cent of the body weight of the European mink. Tarenetzky[5] gave the length of the intestinal canal of a European mink as 560 per cent of the body length; in a European polecat, it was 630 per cent.

Bronn[6] gave a ratio of intestinal length to body length of 4:1 for an ermine *(Mustela erminea)*. Among 25 European polecats, he found the ratio to be 3.8:1 to 8.3:1.

Materials and Methods

The mink used in this study were supplied by The Coöperative Fur Animal Disease Research Laboratory, State College of Washington and The U. S. Department of Agriculture, Bureau of Animal Industry, at Pullman, Wash. A few animals were 1 year of age or older. Some of the mink, some were 3 months old while the majority were about 6 months of age, were embalmed for detailed dissection, but the majority were dissected in the fresh condition.

The lengths of the three parts of the duodenum were measured *in situ* with calipers. The length of the combined jejuno-ileum from the duodenojejunal flexure to the ileocolic junction was measured by transecting the ileocolic junction and freeing the gut from its mesentery so that it could be laid straight along a meter stick and measured to the nearest centimeter. The pancreas, liver, and gall bladder were excised and weighed. The longitudinal diameter of the gall bladder was measured with calipers; the volume of bile was ascertained by aspiration with a needle and syringe. Vinyl acetate casts of the pancreatic and biliary duct systems were produced by inserting a small, blunt needle into the major duodenal papilla and injecting the liquid vinyl acetate. The tissues were then macerated by subjecting them to concentrated hydrochloric acid, leaving the vinyl acetate casts intact.

The combined length of the colon, the rectum, and the anus was measured *in situ* with calipers. The anal sacs were dissected free and excised together with the glandlike structure around the neck of each sac. The secretion was expressed as the anal sacs were flattened against a piece of filter paper. The lengths and widths of the flattened organs were measured; then the sacs were opened, washed in physiological saline solution, blotted dry and weighed.

Results

The Small Intestine.—The first part of the duodenum lacks a definite sigmoid loop. It extends anterad and slightly dorsad approximately 1 cm. from the pylorus. The first duodenal flexure turns abruptly dextrad and then assumes a posterior direction. The right border of the lesser omentum is attached to the first flexure. The second part of the duodenum continues in a posterior direction, slightly ventrad and to the right of the midline. Dorsally, it is related to the caudate lobe of the liver and the right kidney; ventrally, it is related to the greater mass of the small intestine. The major duodenal papilla projects from the dorsal aspect into the lumen of the anterior quarter of the second part. A minor papilla may project into the duodenum slightly anteromedial to the major papilla. At the level of the fifth lumbar vertebra, the duodenum continues into the second duodenal flexure which again reverses the direction of the gut and leads into the third part of the duodenum. The third part runs anterad from the second flexure close to the median plane, ending at the duodenojejunal flexure which is located immediately posterior to

From a thesis submitted in partial fulfillment of the requirements for the degree of master of science in veterinary medicine, State College of Washington, Pullman. The author is now in the Department of Veterinary Pathology, School of Veterinary Medicine, Oklahoma A. and M. College, Stillwater.

the root of the great mesentery. The third part of the duodenum is related on the right to the terminal part of the ileum, on the left, to the colon. A large mesenteric lymph node is related to the duodenojejunal flexure.

The second part of the duodenum is slightly less than twice as long as the third part. The loop formed by the two parts surrounds the right limb of the pancreas. The mesoduodenum encloses the right limb of the pancreas and suspends the second and third parts of the duodenum. A duodenocolic fold continues from the mesoduodenum around the third part of the duodenum to the mesocolon.

From the duodenojejunal flexure, the jejunum enters into the formation of a coiled mass. Some of the coils contact the visceral surfaces of the liver and the stomach for a variable distance. Anterolateral coils on the right side contact the second part of the duodenum. On the left side, the coils are related to the visceral surface of the ventral end of the spleen. The jejunal mass rests on the ventral abdominal wall enclosed ventrally and laterally by a capacious greater omentum. Dorsally, the jejunum is related to the kidneys, the adrenal glands, the ovaries, the cornua uteri, the duodenum, the pancreas, the ileum, and the colon.

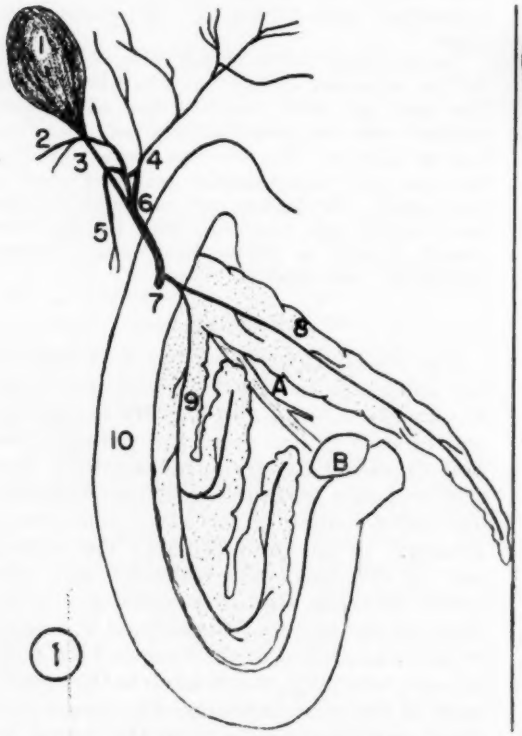

Fig. 1—Reproduction of vinyl acetate casts of the biliary and pancreatic ducts of the mink. Ventral view. x 1⅓.

1) Gall bladder; (2) duct from the right medial and right lateral lobes of the liver; (3) cystic duct; (4) duct from the quadrate, left medial, and left lateral lobes of the liver; (5) duct from the caudate lobe of the liver; (6) common bile duct; (7) major duodenal papilla; (8) duct from the left limb of the pancreas; (9) duct from the right limb of the pancreas; (10) duodenum; (A) portal vein; (B) mesenteric lymph node.

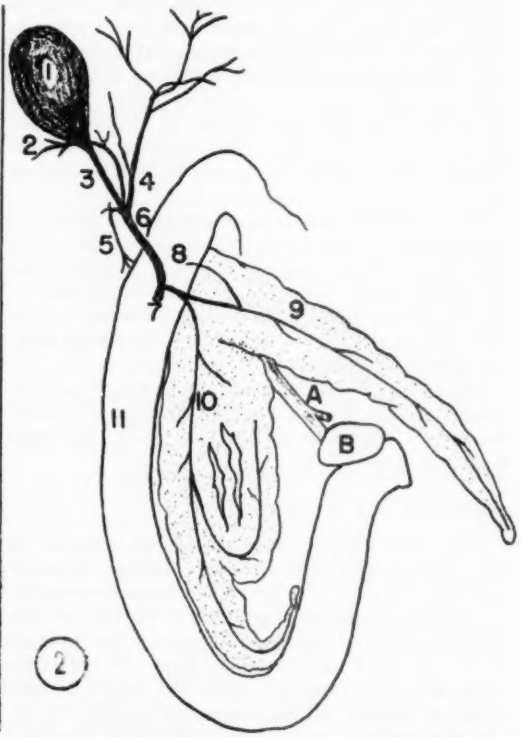

Fig. 2—Reproduction of vinyl acetate casts of the biliary and pancreatic ducts of the mink showing variations from the ducts in figure 7. Ventral view. x 1⅓.

1) Gall bladder; (2) duct from the right medial and right lateral lobes of the liver; (3) cystic duct; (4) duct from the quadrate, left medial, and left lateral lobes of the liver; (5) duct from the caudate lobe; (6) common bile duct; (7) major duodenal papilla; (8) minor duodenal papilla; (9) duct from the left limb of the pancreas; (10) duct from the right limb of the pancreas; (11) duodenum; (A) portal vein; (B) mesenteric lymph node.

There is no grossly recognizable junction of the jejunum with the ileum. A rough approximation might be made at the anastomosis of the ileal artery with the final intestinal artery. The terminal part of the ileum extends anterad, related ventrally to the jejunal mass, dorsally to the pancreas, and medially to the third part of the duodenum. It curves sinistrad anterior to the anterior mesenteric artery, continuing into the colon on the left side of the median plane.

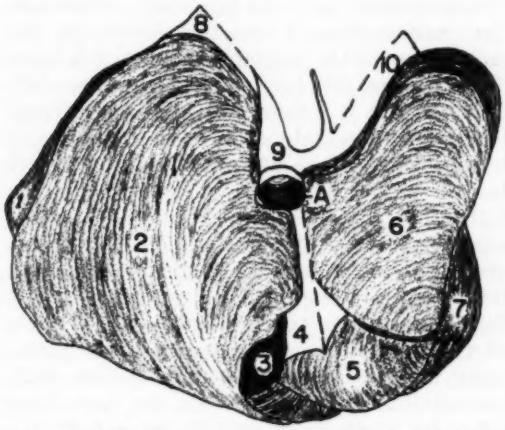

Fig. 3—Parietal surface of a mink's liver hardened in situ. x 2.
1) Right lateral lobe; (2) right medial lobe; (3) gall bladder; (4) falciform ligament; (5) quadrate lobe; (6) left medial lobe; (7) left lateral lobe; (8) right lateral ligament; (9) coronary ligament; (10) left lateral ligament; (A) posterior vena cava.

In 106 mink, ranging from 281 to 425 mm. in body length, the duodenum ranged from 74 to 139 mm. in length with a mean of 106 ± 15 mm. The combined length of the jejuno-ileum ranged from 93 to 150 cm. with a mean of 120 ± 13 cm.

The Pancreas.—The pancreas is an extensive organ in the mink. The right limb follows the second and third parts of the duodenum enclosed in a continuation of the mesoduodenum. This limb also sends an extension into the space between the duodenojejununal flexure on the left and the second part of the duodenum on the right, continuing along the portal vein and often contacting the large mesenteric lymph node located at the duodenojejunal flexure. The dorsal surface of the right limb is related to the posterior vena cava, the right and left renal vessels, the aorta, the caudate lobe of the liver, the right kidney, and the right adrenal gland. The ventral surface is related chiefly to the intestinal mass.

The left limb of the pancreas is very long. It extends posterosinistrad dorsal to the visceral surface of the stomach and then medial to the spleen, the apex contacting the left lateral abdominal wall. The left limb is enclosed in the mesentery which connects the mesoduodenum with the gastrosplenic ligament. This limb is distinctly triangular in cross section. Its dorsal surface is related to the portal vein, the left kidney, and the left adrenal gland; its ventral surface is related to the colon and the intestinal mass.

The color of the pancreas is a light pink; the normal texture of the organ is moderately soft. The weight of the pancreas ranged from 2.0 to 4.8 Gm. with a mean of 3.0 ± 0.5 Gm. in 73 mink ranging from 400 to 1,450 Gm. in body weight.

A large duct with many radicles drains each limb of the pancreas. The two ducts converge to form a single pancreatic duct which joins the ductus choledochus just before the termination of the latter at the major duodenal papilla. An accessory pancreatic duct may extend from the duct which drains the left limb to the minor

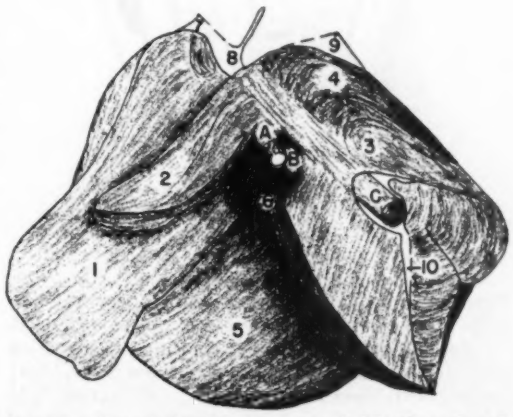

Fig. 4—Visceral surface of a mink's liver hardened in situ. x 2.
1) Left lateral lobe; (2) papillary process of the caudate lobe; (3) caudate process of the caudate lobe; (4) right lateral lobe; (5) right medial lobe; (6) common bile duct; (7) left lateral ligament; (8) coronary ligament; (9) right lateral ligament; (10) hepatorenal ligament; (A) hepatic artery; (B) portal vein; (C) posterior vena cava.

duodenal papilla. Figures 1 and 2 illustrate variations in the pancreatic and biliary duct systems as determined by the preparation of vinyl acetate casts.

The Liver.—The mink possesses a relatively large liver. The weight of the liver ranged from 22.0 to 54.3 Gm. with a mean of 35.4 ± 4.9 Gm. in 76 mink ranging from 400 to 1,450 Gm. in body weight. The liver averaged 4,904 mg. per 100 Gm. of body weight.

The part of the parietal surface of the liver which is related to the diaphragm is strongly convex (fig. 3). The part which is related to the ventral abdominal wall is extensive, the sharp ventral border projecting beyond the costal arch.

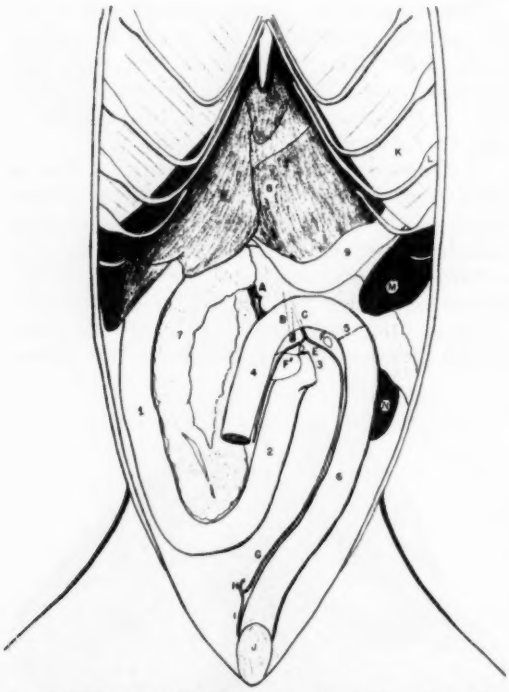

Fig. 5—Deep dissection of the abdominal viscera. Mesenteric attachments removed. x 0.8. 1) Second part of the duodenum; (2) third part of the duodenum; (3) jejunum (cut); (4) ileum; (5) ileocolic junction; (6) colon; (7) pancreas; (8) liver; (9) stomach; (A) portal vein; (B) anterior mesenteric artery; (C) ileocolic artery; (D) ileal artery; (E) right colic artery; (F) mesenteric lymph note at the ileocolic junction; (F') mesenteric lymph node at the duodenojejunal flexure; (G) left colic artery; (H) posterior mesenteric artery; (I) anterior hemorrhoidal artery; (J) urinary bladder; (K) diaphragm; (L) twelfth rib; (M) spleen; (N) left kidney.

Six lobes are presented for description. The left lateral lobe is the largest. Its visceral surface contacts the parietal surface of the stomach and the papillary process of the caudate lobe. There is a notch for the esophagus on the dorsal border of this lobe. The visceral surface of the smaller left medial lobe is related to the left lateral lobe. The quadrate lobe is medial to the left medial lobe, contacting it and the left lateral lobe. There is a deep fossa vesicae felleae in the quadrate lobe. The visceral surface of the right medial lobe, which is second in size, is related to the quadrate lobe, the gall bladder, the cystic duct, the left lateral lobe, the first and second parts of the duodenum, a few coils of the jejunum, and the right lateral lobe. The right lateral lobe is smaller than the preceding lobe. Its visceral surface is related to the second part of the duodenum, a few coils of the jejunum and the caudate process of the caudate lobe. The caudate lobe is well developed. The papillary process lies along the anterior half of the lesser curvature of the stomach. The caudate process is large and indented by a deep renal impression which receives the anterior third of the right kidney. This process also contacts the right dorsal aspect of the anterior half of the second part of the duodenum. Figure 4 illustrates the visceral surface of the liver.

The portal fissure is located at the convergence of the caudate and right lateral lobes. The hepatic artery crosses the dorsomedial part of the fissure where it divides into its lobar branches. The portal vein is located in the middle of the fissure while the hepatic duct occupies a ventral position. The posterior vena cava is deeply embedded in the dorsomedial aspect of the caudate lobe of the liver and continues through the right lateral lobe in a similar position. It receives an hepatic vein from the caudate lobe as it passes through this lobe and three or four more hepatic veins just before it enters the foramen venae cavae.

The falciform ligament has two parts. The major part emerges between the left medial and quadrate lobes; the minor part comes out between the right medial and quadrate lobes. The minor part also continues to the gall bladder. The major part surrounds the posterior vena cava and be-

comes continuous with the coronary ligament. The round ligament occupies its usual position in the free edge of the falciform ligament. The coronary ligament encloses the posterior vena cava as the vessel emerges from the dorsal aspect of the right lateral lobe. This ligament also surrounds the abdominal part of the esophagus. On each side it is continuous with a lateral ligament. The right lateral ligament and the left lateral ligament are about equally developed. They extend from the dorsal borders of their respective lobes to the diaphragm. The hepatorenal ligament extends from the dorsoanterior aspect of the renal impression, mainly from the medial side, around the posterior vena cava to the right kidney.

The reproductions of vinyl acetate casts in figures 1 and 2 illustrate the distribution of the larger bile ducts. The largest duct drains the left side of the liver; it receives ducts from the left lateral, left medial, and quadrate lobes. This large duct together with a duct from the right side of the liver and another duct from the caudate lobe join the cystic duct. They do not form a definite single hepatic duct but rather all three join with the cystic duct to form the common bile duct. They may anastomose before they finally join. The common bile duct is joined by the pancreatic duct and then terminates at the major duodenal papilla.

The gall bladder is located in the fossa vesicae felleae of the quadrate lobe overlapped by the right medial lobe. Depending on its distension, the gall bladder may slightly separate the two lobes. It contains approximately 0.5 to 1.0 cc. of bile. The mean length of the gall bladder was found to be 21 $\pm$ 4 mm. in a series of 76 mink. The cystic duct runs between the quadrate and right medial lobes, then deep to the latter where it joins the three hepatic ducts to form the common bile duct.

The Colon and Rectum.—The cecum is absent. The ileocolic junction occurs after the ileum executes its turn anterior to the anterior mesenteric artery, the actual mucosal junction taking place to the left of the median plane. A topographically accurate ascending colon or transverse colon can not be differentiated in the mink. A constantly occurring mesenteric lymph node related to the first part of the right colic artery may be used as an approximate guide to the locus of the ileocolic junction (fig. 5). The mucosal junction of the ileum and the colon is clearly marked. Where the villi of the ileum terminate the velvety appearance which they impart to the mucosa is replaced by boldly outlined longitudinal folds in the colon. The cessation of the villi and the continuation of the crypts of Lieberkuhn are easily discernible with a magnification of 15 diameters.

In its initial part, the colon is related dorsally to the posterior vena cava, the ventral aspect of the left limb of the pancreas, the left phrenicoabdominal vein, and the left adrenal gland. Farther posteriorly, it contacts the medioventral aspect of the left kidney and the left renal vessels. It deviates slightly to the left, crosses the posterior pole of the left kidney and then curves to the right, coming to the midline at the brim of the pelvis where it continues into the rectum. Ventrally, the colon is related to the mass of the small intestine and, in its terminal part, the urinary bladder. There are no haustra coli or taenia coli. When the gut is empty, the colon has approximately the same diameter as the small intestine—about 5 or 6 mm. The diameter increases slightly as the colon approaches the rectum.

The rectum does not have a capacious ampulla recti. The peritoneal part reaches

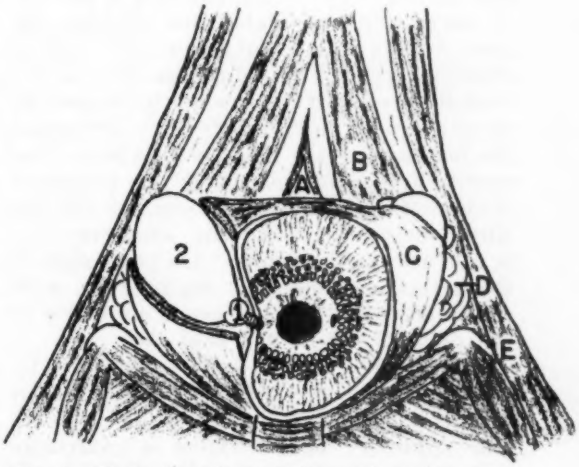

Fig. 6—Posterior view of the anal region of a mink dissected to show the position of the anal sacs. x 2.

1) Glandlike structure around the neck of the left anal sac; (2) left anal sac; (A) suspensory ligament of the anus; (B) coccygeus lateralis muscle; (C) sphincter ani externus muscle; (D) fat; (E) abductor cruris caudalis muscle.

a plane perpendicular to the second coccygeal vertebra. In the male, the rectum is related ventrally to the neck of the urinary bladder and the prostate gland; in the

Fig. 7—Sagittal section of the rectum and the anus of a mink. x 2.
1) Rectum; (2) columnar zone of the anus; (3) intermediate zone of the anus; (4) opening of the duct of the anal sac; (5) cutaneous zone of the anus; (A) rectococcygeus muscle and the suspensory ligament of the anus; (B) sphincter ani externus muscle.

female, it is related ventrally to the vagina, the cervix uteri, and the corpus uteri.

In a series of 106 mink, ranging from 281 to 425 mm. in body length, the combined length of the colon, rectum, and anus ranged from 75 to 127 mm. with a mean of 101 ± 11 mm. The ratio of intestinal length to body length ranged from 3.60:1 to 4.38:1 with a mean ratio of 3.97:1.

The Anus.—The extent of the anus is best indicated by the mucosa (fig. 6 and 7). A narrow intermediate zone divides the anal mucosa into a cutaneous zone and a columnar zone. The cutaneous zone is devoid of hair and characterized by numerous elevations and minute pits. The elevations are larger near the intermediate zone. The longitudinal columns, which are supposed to characterize a columnar zone, are not distinct; therefore, a definite anorectal line is not always discernible. On each side of the cutaneous zone, near its junction with the intermediate zone, there is an orifice of the duct of one of the anal sacs.

The greater part of each anal sac is located between the sphincter ani externus and the sphincter ani internus muscles. The sphincter ani externus is extremely thin where it covers the dorsolateral surface of the anal sac. At this point, muscle fibers are only faintly visible (fig. 6, C). Ventrally and, to a lesser degree, dorsally the sphincter ani externus is thick and fleshy. By tensing an apparent median fold extending from the second and third coccygeal vertebrae to the anus a few muscle fibers may be seen in the anterior part of the fold. These fibers represent the rectococcygeus muscle. The posterior part of the fold flares out; this part could be considered the remnant of the suspensory ligament of the anus.

A white, lobulated glandlike structure surrounds the neck of each anal sac. It is not to be confused with a variable amount of dark-colored fat which also surrounds the neck. A short duct connects the anal sac with the orifice in the cutaneous zone of the anal mucosa. The fundus of the sac extends beyond the anterior border of the thin part of the sphincter ani externus into the fat-filled ischiorectal fossa. It is related dorsolaterally to the abductor cruris caudalis muscle, medially to the coccygeus lateralis muscle, anteriorly to the coccygeus medialis muscle, and ventrally to the obturator internus muscle. The wall of the anal sac is white and folded along its longitudinal axis. The number of folds is modified by the distension of the organ with its thick, yellowish white secretion.

In 19 mink, ranging from 420 to 1,450 Gm. in body weight, the mean weight of each anal sac was 114 ± 26 mg., the mean length was 17 ± 2 mm., and the mean width 8 ± 2 mm. There was little significant difference between right and left anal sacs.

DISCUSSION

The postmortem measurements of excised organs do not necessarily reflect the dimensions of the same organs during life. Factors such as the manner in which an animal was killed, the method used in removing an organ, hemorrhage, and the time since death may be responsible for significant variations. Consistent techniques were employed in measuring organs in an effort to insure a constant factor in measuring errors.

Four out of a series of 77 pancreas were abnormally hard and small, weighing 0.7, 1.5, 1.5, and 1.6 Gm., respectively. Microscopic examination of tissue sections revealed a chronic fibrotic pancreatitis. These weights were not incorporated into the data on the weights of the pancreas. The liver averaged 4,904 mg. per 100 Gm. or 4.9 per cent of the body weight. Wood's [1] average of 5.1 per cent is slightly greater. Schutze's [4] finding that the liver of the European polecat constituted 4.6 per cent of the body weight compares favorably

with the above figures. The volume of bile in the gall bladder and opening of the common bile duct given by Neseni [2] for the European polecat were similar to the same structures in the North American mink.

The absence of a cecum in the North American mink is in accord with other members of the family Mustelidae. The lack of topographically accurate ascending and transverse colons necessitates reference to the colon as a single entity. The occurrence of a constant mesenteric lymph node adjacent to the ileocolic junction provides a convenient landmark for approximating the extent of the ileum and the colon. The mean ratio of the intestinal length to body length was 3.97 : 1 in the North American mink, essentially the same ratio as Bronn gave for the ermine. The ratios of 4.6 : 1 for the European mink and 5.6 : 1 for the European polecat given by Neseni are greater.

SUMMARY

1) The midgut and hindgut of the mink were described.

2) A major duodenal papilla projects into the anterior one fourth of the second part of the duodenum. A minor papilla may be present slightly anteromedial to the major papilla. The duodenum has a mean length of 106 mm. There is a large mesenteric lymph node at the duodenojejunal flexure. The jejunal mass rests on the ventral abdominal wall. The mean length of the jejuno-ileum is 120 cm.

3) The pancreas consists of an extensive right limb which lies in the duodenal loop and a long triangular left limb, the apex of which contacts the left lateral abdominal wall. The organ is light pink and has a moderately soft texture. A large pancreatic duct joins the ductus choledochus. An accessory pancreatic duct may empty at the minor duodenal papilla. The mean weight of the pancreas is 3.0 Gm.

4) The relatively large liver presents six lobes for description. The parietal surface is strongly convex. Three large bile ducts, one from the left side, one from the right side, and one from the caudate lobe join the cystic duct to form the ductus choledochus. The latter is joined by the pancreatic duct and then terminates at the major duodenal papilla. The liver has a mean weight of 35.4 Gm. or about 4.9 per cent of the body weight.

5) The cecum is absent. The colon lies entirely to the left of the median plane. A mesenteric lymph node related to the first part of the right colic artery may be used as an approximate guide to the ileocolic junction. The mean combined length of the colon, rectum, and anus is 101 mm.

6) The ratio of intestinal length to body length ranged from 3.60 : 1 to 4.38 : 1 with a mean ratio of 3.97 : 1.

7) The anal sacs are located between the internal and external sphincters of the anus. A small glandlike structure surrounds the neck of each anal sac. A short duct leads to the orifice in the lateral part of the cutaneous zone of the anal mucosa. The mean weight of the anal sac is 114 mg., the mean length 17 mm., and the mean width 8 mm.

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Experimental Infection of Dogs, Ferrets, Chinchillas, and Hamsters with Vesicular Stomatitis Virus

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NATURAL outbreaks of vesicular stomatitis have been reported among horses and cattle, rarely among swine. This malady is characterized by pyrexia, vesiculation, and subsequent ulceration of the oral mucosa and sometimes of the skin of the coronary band, interdigital space, udder, and teats.

The similarity of the lesions of vesicular stomatitis (VS) to foot-and-mouth disease of cattle and swine and to vesicular exanthema of swine may confuse and complicate its diagnosis.

During the outbreak of VS in Wisconsin in 1949 [3] many questions arose; among them was the degree of susceptibility of various species of laboratory as well as other domestic animals to the infection. For this reason, susceptibility trials have been made in this laboratory on sheep, goats, dogs, guinea pigs, hamsters, ferrets, white rats, mice, chinchillas, and chickens.[7] The present report will deal chiefly with the experiments on susceptibility to vesicular stomatitis virus (VSV) of species previously not investigated, namely, dogs, hamsters, chinchillas, and ferrets, whereas the other animals mentioned above were employed in comparative tests.[7] Wagener[15] investigated the pathogenicity of VSV for cattle, hogs, horses, sheep, goats, cats, white mice, rabbits, chickens, pigeons, white rats, and wild rats. He found all species, with the exception of cats, rabbits, white mice, chickens, and pigeons, to be susceptible to VSV infection. Cotton [1] was not successful in transmitting the infection to sheep, rabbits, white rats, and white mice. Mohler,[8] Cotton,[1] and Olitsky, Traum, and Schoening,[9,10] reported the transfer of VSV infection to hogs. Olitsky [11] was not able to infect rabbits. Hanson, Rasmussen, Brandly, and Brown [5] described four infections of man with VSV. Frank, Appleby, and Seibold [4] obtained infection by intracerebral inoculation of horses, cattle, and sheep with the VSV. Encephalitis was manifested by all.

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Paper NS 120 from the Department of Veterinary Science, University of Wisconsin, published with the approval of the director of the Wisconsin Agricultural Experiment Station.

Appreciation is expressed to Dr. R. P. Hanson for valuable suggestions during the course of this study.

MATERIALS AND METHODS

The vesicular stomatitis virus used was a strain of the New Jersey type isolated from an affected cow during an epizoötic in the northwestern part of Wisconsin in 1949.[3] Their procedure of cultivation, isolation, and manipulation was as follows: The mucosal tissue of freshly ruptured vesicles on the oral mucosa of a cow was collected and triturated with physiological saline solution, then penicillin and streptomycin were added to a final concentration of 100 units each per milliliter and the mixture was allowed to stand at room temperature for thirty minutes. The suspension was cultured on blood agar, spun for fifteen minutes in an International centrifuge at 3,500 r.p.m. and the supernatant fluid injected in 0.1-ml. quantities into the allantoic chamber of 8-day-old embryonating chicken eggs. Material for all trials was used only when found to be free from bacteria. The eggs were candled twice daily during subsequent incubation in order to detect death of embryos. All eggs in which the embryos died between the twenty-fourth and ninety-sixth hour after inoculation were chilled for six to twelve hours at 6 to 8 C.; the allantoic fluid was harvested aseptically and tested for sterility. Sterility tests failed to reveal bacterial growth. The titer of pooled fluid from several eggs was estimated by the following method: Serial tenfold dilutions of the fluid were prepared and 0.1 ml. of each dilution was injected into the allantoic chamber of each of a series of four, 9-day-old embryonating eggs. Deaths of the embryos occurring during the subsequent forty-eight to ninety-six hours were recorded and the results calculated according to the Reed and Muench [12] formula.

Several additional egg passages of the virus were accomplished and the virus-bearing fluids stored at —25 C. for future use.

All animals used in the experiments were inoculated with fluids originating from the eighth serial passage in eggs. Inoculation was by the following routes: intramucosal, intradermal, intravenous or intracardial, intracerebral, corneal, and intranasal. The virus was injected with a sterile syringe and needle in all except where it was applied directly to the scarified cornea or instilled into the nares.

Intracerebral inoculation was into the frontal lobes. In chinchillas and hamsters, 23- or 27-gauge

hypodermic needles were used for piercing the calvarium, whereas in dogs and ferrets preliminary trephination with aseptic precautions was necessary. All intracerebral and intracardial injections of the animals were performed under ether or nembutal narcosis.

During the week prior to inoculation, normality was ascertained by clinical examination and body temperature determination. Blood was collected from each animal at this time for later testing for VSV neutralizing antibodies. The results of antibody studies will be presented elsewhere.

RESULTS

Dogs (Canis familiaris).—These trials were with two groups of 3 dogs each, the animals of one group being 10 weeks old, those of the second, 1 year old. One dog of the younger group was injected intracerebrally (0.3 ml.), another intravenously (3.0 ml.), and the third, into the mucosa of the mouth (0.5 ml.). The protocol was the same for the older group except that the dosage was 0.5 ml., intracerebrally, 5 ml. intravenously, and 0.5 ml., intramucosally. The inoculated animals were observed subsequently for fourteen days to detect any formation of vesicles of the oral cavity of the body surface, of pyrexia, loss of appetite, abnormal activity, deranged consciousness, or disorder of the respiratory or alimentary tracts. Abnormal signs were not observed in any of the animals.

Hamsters (Cricetus auratus).—Sixteen animals 4 months of age and 14, 4 weeks of age were studied. Following intracerebral inoculation (0.1 ml.), each of 4 hamsters in the older age group and 4 in the younger age group died on the second day with signs of general paralysis. Each of the 4 animals in both age groups exposed by nasal instillation (0.2 ml.) died six days after having developed clinical signs of pneumonia, nasal discharge, dyspnea, and high body temperature. Intracardial exposure (0.3 ml.) of 4 older animals and 3 younger animals failed to produce any abnormality.

Four, 4-month-old hamsters were entirely refractory to intraperitoneal inoculation (0.5 ml.) and only 1 of 3, 5-week-old hamsters developed posterior paralysis and died following this exposure.

Chinchillas (Chinchilla lanigera).—The susceptibility of chinchillas to VSV infection was studied among 8 1-year-old animals. Intracerebral inoculation (0.1 ml.) of 2 animals resulted in death of both on the third day. After the second day, signs of incoördination, clonic and tonic seizures, general convulsions, paraplegia, and general paralysis were observed. The intranasal instillation (0.3 ml.) of VSV into 2 animals caused the development of fatal pneumonia. Anorexia, pyrexia, sneezing, bilateral seromucous nasal discharge, and prostration were observed on and subsequent to the second day of exposure. During the next five days there was progressive dyspnea and the temperature dropped to subnormal where it remained until death on the seventh to eighth day after inoculation. The clinical diagnosis of pneumonia was confirmed by gross and histopathological findings and by virus isolation from the affected lungs.

In 2 chinchillas exposed simultaneously in the skin of the foot pad (0.1 ml.) and the mouth mucosa (0.1 ml.), there was no local effect but from the sixth day on slight paraplegia of the hind quarters, indicated by locomotor ataxia, was apparent. In the meantime, involuntary tonic and clonic spasms of all the limbs as well as intermittent tremor of the head, torticolis, or opisthotonus were noticed. The body temperature was within normal limits during the first four days but became markedly elevated on the fifth and sixth. On the seventh and eighth days, it dropped to a subnormal level. Death was preceded by general paralysis.

Inoculation of 2 chinchillas into the hind foot pads alone failed to induce any local or general reaction.

Ferrets (Putorius furo, Linn).—Sixteen ferrets, between 6 to 8 months of age, were inoculated intradermally, intramucosally, intracerebrally, intracardially by corneal scarification and intranasal instillation.

On the fourth and fifth day following the inoculation into the oral mucosa, 4 animals developed well-marked progressive lesions of the tongue, dental pads, lips, and skin of the foot pads. Anorexia, restlessness, pyrexia, and salivation were associated signs. Observable local lesions consisted of small erosions which during the next day extended rapidly to involve large areas of the dental pads, buccal mucosa, and dorsum of the tongue to leave a denuded, bleeding submucosa. A slight nasal discharge was also present. Foot pad changes, which appeared simultaneously with oral lesions, consisted of redness,

swelling, and the formation of vesicles of the pads. The vesicles ruptured after a short time and left denuded, red surface. The animals appeared to move only with reluctance and difficulty. Prehension and mastication of food was impaired with resultant loss of body weight. The mucosal erosions and foot pad ulcerations were covered by freshly growing epithelium within seven to ten days. The repair was equally as rapid as that which has been observed by us and others in cattle. When the appetite returned the animals regained a good appearance and normal physical condition.

Similar signs and lesions developed after VSV was instilled into the nares of 4 ferrets. Lesions of the oral mucosa were not as extensive as after mucosal injection but foot pad ulcerations were equally pronounced.

Intracerebral injection (0.2 ml.) of 2 animals resulted in general signs including anorexia, pyrexia, fatigue, and vesicle formation of the mouth mucosa and foot pads. One animal died after general paralysis on the tenth day but the second ferret recovered.

Two ferrets injected intracardially with 0.5 ml. of virus fluid developed pyrexia, anorexia, weakness, swelling, and vesicles of the foot pads. After rupture and ulceration of the foot lesions, healing proceeded without complications. Improvement in general health was observed during the few days immediately following vesiculation.

Inoculation of virus into the foot pads of 2 animals was followed by marked swelling of the surrounding area, three to four days after exposure. Some vesicles developed and ruptured within the next several hours leaving ulcerated areas. Drying and healing of the areas was quite rapid. Generalization of the infective process was not observed although there was a slight pyrexia.

Finally, 2 additional ferrets were infected by applying the virus to the scarified cornea. Slight conjunctivitis and keratitis with ulceration developed, but other areas or tissues of the body were not visibly involved. The body temperature remained normal during the ensuing few weeks.

DISCUSSION

In the experiments of others,[1, 4, 8, 9, 10, 15] the New Jersey type of VSV manifested differential tissue affinities for animals of different ages. The same occurred in our experiments with the majority of young guinea pigs, hamsters, and mice.

TABLE 1—Species Susceptibility to Vesicular Stomatitis Virus Infection by Different Routes of Inoculation

	Intracerebral	Intradermal	Intravenous or Intracardial	Intranasal
Hamster	+ + + +	?	+ + + +	+ + + +
Ferret	+ + + +	+ + + +	+ + + +	+ + + +
Chinchilla	+ + + +	+ +	?	+ + + +
Dog	− − − −	− − − −	− − − −	?

Ferrets in this experiment showed a tendency toward pronounced vesiculation similar to that observed by the above in horses, cattle, swine, and guinea pigs. Vesiculation was not seen in hamsters or chinchillas.

The incubation period varied in relation to the route of exposure, the species, and dosage. In ferrets and in swine, the incubation period was three to five days after intradermal, intracerebral, intravenous, intracardial, intramucosal, and intranasal instillation. In mice, guinea pigs, hamsters, and chinchillas, signs appeared within forty-eight hours after intranasal instillation or intramucosal or intracerebral injection. Rabbits did not show any signs of infection to any route of exposure or volume of inoculum. They developed antibodies although only after large volumes of virus-containing fluids were injected parenterally.

The course of the disease varied also. Horses, cattle, and swine invariably survived exposure to VSV by all routes and recovery was complete within eight to fourteen days after vesiculation was first noticed. Ferrets usually survived infection via intracerebral inoculation. In mice, hamsters, and chinchillas the course of the disease was short with death occurring in most instances, in one, two, and six days. With several exceptions, the results of our work with the Wisconsin strain agree with that or other strains by various investigators. The differences were in the susceptibility of the rabbit, sheep, goat, and cow. Olitsky [8] was able to produce encephalitis in horses, cattle, and sheep, whereas our results were negative.[7] Wagener [15] induced vesiculation of the oral mucosa in 1 of 4 inoculated sheep and in both of 2 goats.

No one has succeeded in infecting dogs but in the outbreak of vesicular stomatitis in Wisconsin in 1949 [3] and in Colorado in 1948 [6] there were field reports of oral vesiculation among dogs.

SUMMARY

Susceptibility to vesicular stomatitis virus (VSV) of domestic and laboratory species to different routes of exposure was investigated. Added to the group of susceptibles were three new species, namely, the hamster, chinchilla, and ferret, all being easily infected by intranasal instillation. The three proved to be sensitive also to intracerebral exposure. Hamsters and chinchillas developed fatal encephalitis while some of the ferrets recovered. The ferrets developed lesions on the foot pads after intracerebral inoculation. The relative susceptibilities to VSV of the four newly tested species may be summarized as follows. The degree differences being expressed by positive reactions from + + to + + + +.

All three species were susceptible to intranasal instillation. The hamsters and chinchillas succumbed after infection by this route whereas the ferrets survived. Neither hamsters nor chinchillas showed signs of generalization of the infectious process such as occurred in ferrets.

Dogs were resistant to intramucosal, intravenous, and intracerebral infection with the Wisconsin strain of VSV. Their experimental exposure had not been reported previously.

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Studies on an *in Ovo* Neutralization Test for Distemper

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SINCE the classic works of the English investigators, Laidlaw and Dunkin (Laidlaw [9]), research on distemper has been mainly concerned with pathological studies and the development and evaluation of prophylactic and therapeutic measures. Until recently there has been relatively little basic research on the immunological and epizoötiological aspects of the disease. Perhaps the main reason is the lack of effective research tools, such as serological tests.

The adaptation of distemper virus (DV) to eggs by Haig,[7] Cabasso and Cox,[6] and West[14] made possible many new approaches to the study of distemper. For example, it was found that the embryonating egg could serve as a test animal in a virus-serum neutralization procedure.

Cabasso and Cox [6] demonstrated by egg inoculation that the egg-grown virus was neutralized *in vitro* by serum from hyperimmunized ferrets, dogs, and guinea pigs. Cabasso *et al.*[4] demonstrated high levels of neutralizing antibodies in pooled serum samples from puppies vaccinated one month previously with a single dose of the egg-adapted virus.

Recently, Cabasso and Cox [5] demonstrated by cross-neutralization tests in eggs and ferrets the serological relationship between the Lederle strain of egg-adapted DV and an egg-adapted strain which originated from a case of canine encephalitis. They also found that distemper immune dog serum and commercial "hardpad" antiserum neutralized the egg-adapted encephalitis strain to approximately the same dilution (1: 64), whereas immune ferret and dog serum neutralized the encephalitis strain at a slightly higher dilution (1: 256).

Belcher [2] conducted neutralization tests in eggs with two strains of egg-adapted DV (Haig and FXNO-Wisconsin strains), using serum collected from ferrets three and five weeks after inoculation with the homologous virus. She found serum titers ranging from 1: 83 to 1: 3,262 at three weeks and from 1: 25 to 1: 246 at five weeks.

The present report deals with the development of a quantitative neutralization test, using the embryonating egg as the test animal, and the results of such a test on mink and ferret serum collected under various conditions of exposure to distemper virus.

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MATERIALS AND METHODS

Virus.—Haig's Onderstepoort (HG) strain of egg-adapted DV from the eighty-fifth and eighty-sixth egg passages was used in these experiments in the neutralization tests and as a vaccine. This virus was found to be avirulent for mink and ferrets. When inoculated onto the chorioallantoic membrane (c.a.m.) of developing chicken embryos, this virus causes opaque, grayish plaques, and thickening and edema of the membrane.

The virus suspensions for use in the neutralization test were prepared as follows: A frozen stock suspension of HG-infected membranes was thawed and inoculated onto the c.a.m. of 7-day-old chicken embryos. All egg inoculations were made on the c.a.m. by a modification of the "dropped membrane" technique. Following incubation at 37 C. for an additional seven days, the membranes were harvested, pooled, and ground in a Waring blendor with an equal quantity of sterile inactivated horse serum. The suspension was then centrifuged at 6,000 r.p.m. for thirty minutes in an International refrigerated centrifuge at 1 C. The supernatant fluid was removed, mixed, distributed in 2-cc. ampoules, sealed, and stored in a dry-ice chest until used.

The vaccines used in these experiments consisted of HG-infected c.a.m. inoculated, incubated, and harvested as described above, then ground with sufficient nutrient broth (Difco) to make a 20 per cent suspension, distributed in ampoules, sealed, and frozen until used. A portion of each lot of vaccine was tested for bacteriological sterility and for viability of the virus by inoculation into eggs.

Diluent.—Ten per cent inactivated horse serum in physiological saline solution was used in the neutralization tests as the diluent for serum and virus preparations. Antibiotics were added to the diluent in the following concentrations: crystalline penicillin G, 1,000 units per milliliter, and dihydrostreptomycin sulfate, 100 μg per milliliter.

Collection and Storage of Serum.—The mink and ferrets were bled by cardiac puncture under ether anesthesia. Dogs were bled from the cephalic vein. The serum thus obtained was stored in a freezer at —30 C. until used. All samples were tested within eight months from the time they were collected.

Animals.—In one experiment 45, 6-month-old mink from the stock colony at the Fur Animal Disease Station were used. Twenty-three ferrets, naturally infected with distemper during an out-

break of the disease in the ferret colony, were also used in this experiment to provide a source of exposure to part of the mink. In another experiment which was carried out one year later, 33, 5- to 6-month-old ferrets from a new stock colony were used. These young ferrets came from a group of unvaccinated breeding stock which was obtained following the distemper outbreak the previous year.

Calculations.—In the virus titrations, the 50 per cent infectivity endpoint (i.d.$_{50}$) was calculated by the method of Reed and Muench [12] in order to determine how many virus i.d.$_{50}$ were contained in the test dose mixed with the serum dilutions. The 50 per cent neutralizing dose (n.d.$_{50}$) was calculated for each serum by the same method. This figure represents the dilution of serum, expressed as the antilog, that would prevent the appearance of lesions in 50 per cent of the eggs inoculated with the serum-virus mixtures. In all cases, the resulting numbers were rounded off to two significant figures. Geometric means were calculated for each group of mink serum titers.

EXPERIMENTAL DATA

Development of a Quantitative Neutralization Test.—(a) Methods of Titrating Virus.—Because the HG virus produces visible plaques on the c.a.m. of infected eggs, the pock-counting technique of Beveridge and Burnet [3] was investigated as a means of estimating the number of infective units in a virus suspension. This method was eventually abandoned because of the tendency of the plaques to become confluent and the excessive variation in the number of lesions in different eggs inoculated with the same dilution.

The HG virus, when inoculated in large quantities, was occasionally found to cause death of the embryos. In our experience, however, this phenomenon was not sufficiently consistent to be used as a criterion of virus titer.

The most satisfactory procedure for titrating the virus appeared to be that of inoculating groups of six to eight eggs each with serial tenfold or fivefold dilutions of virus, ranging from a dilution which produced marked lesions in all eggs to one which produced no lesions. By recording the presence or absence of lesions in each egg inoculated, the 50 per cent infectivity endpoint of each virus suspension could be calculated.

b) Procedures for Preparing Serum-Virus Mixtures.—The serial serum dilution-constant virus procedure was chosen for these tests because many high titer serums, undiluted or in low dilutions, completely neutralized low dilutions of virus.

Early in the work, normal, unheated mink or ferret serum in low dilutions was found to cause death of the embryos when inoculated alone. Heated serum (56 C. for thirty minutes) had no apparent effect on the embryos. Thereafter, all serums to be tested were heated in a 56 C. water bath for thirty minutes prior to the preparation of dilutions.

A minimum of 100 i.d.$_{50}$ of virus per inoculum (0.1 ml.) appeared to be desirable in the serum-virus mixtures because this quantity of virus produced extensive lesions on the c.a.m. to control eggs and yet apparently was not so excessive as to reduce the sensitivity of the test in detecting small amounts of antibody.

c) Effect of Variations in Quantity of Virus on Resulting Serum Titers.—A number of titrations on different ampoules of virus from the same lot showed considerable variation in the virus titer, as much as tenfold in some instances. Because the serum titer is directly dependent upon the amount of virus used in the serum-virus mixtures, a number of titrations of the same serum sample were conducted, using fivefold serum dilutions with different ampoules and varying dilutions of the virus preparation. Each ampoule of virus was titrated in the usual manner. The serum used in these tests was taken from a healthy, adult dog.

Table 1 shows the results of these tests. The results are arranged in ascending order according to the number of virus i.d.$_{50}$ per inoculum used in the tests. Those tests in which the number of virus i.d.$_{50}$ fell below 100 are not included.

TABLE 1.—Effect of Varying Quantities of Virus on Serum Titers

Log virus i.d.$_{50}$	Dilution of virus used in test	No. i.d.$_{50}$ per inoculum	Serum n.d.$_{50}$*
4.1	1:100	130	3,200
3.7	1:30	170	2,500
3.9	1:40	200	630
4.0	1:40	250	2,000
4.1	1:30	430	1,000
3.7	1:10	500	630
4.1	1:10	1,300	400

i.d.—infectivity endpoint; n.d.—neutralizing dose; * expressed as reciprocal of n.d.$_{50}$ dilution.

It can be seen that a tenfold variation in the amount of virus used in the tests (from 130 to 1,300 i.d.$_{50}$ per inoculum) resulted in an eightfold difference in the resulting serum titer (from 1:3,200 to 1:400). It is apparent from these data that for reproducibility of serum titers within a narrower range, the virus titer must be controlled within closer limits.

Technique of the Test.—A quantity of each serum to be tested was heated in a 56 C. water bath for thirty minutes. Then serial tenfold or fivefold

dilutions were prepared in short tubes as follows: In the mink serum titrations, 2: 5 (for low titer and negative control serums), 1: 5, 1: 50, 1: 500, et cetera; for the ferret serum titrations, undiluted (for low titer and negative control serums), 1: 5, 1: 25, 1: 125, et cetera. Five dilutions were usually adequate. Negative control serums included with each series were used undiluted or diluted 2: 5 only.

After completion of the serum dilutions, the rack containing the tubes was placed in a pan of ice water. Two ampoules of prepared virus-containing membrane suspension were then thawed and the contents pooled and mixed. This material was used to prepare a dilution of membranes which, when mixed with an equal quantity of serum, was calculated, on the basis of previous titrations, to contain approximately 100 embryo i.d.$_{50}$ of virus per 0.1 ml., the quantity of inoculum for each egg. This dilution of membranes was then added in equal quantity to each tube of the serum dilutions, giving final serum dilutions of 1: 5, 1: 10, 1: 100, 1: 1,000, et cetera, or 1: 2, 1: 10, 1: 50, etc.

A virus titration was included with each series of tests. This consisted of a series of five tenfold dilutions of virus, beginning with the dilution used in the serum-virus mixtures, added to tubes containing an equal quantity of cold diluent.

After the serum-virus mixtures and the virus dilutions were completed and mixed, they were incubated for twenty-four hours at 5 C., then inoculated onto the c.a.m. of 8-day-old chicken embryos in 0.1-ml. quantities, using six eggs for each serum-virus mixture and six or eight eggs for each virus dilution. Following further incubation for six days at 37 C., the eggs were opened and the c.a.m. examined for the presence of distemper lesions. Results were recorded as + (lesions present) or — (no lesions present) for each egg. Absence of lesions in eggs inoculated with serum-virus mixtures was considered as evidence that the virus had been neutralized by the serum. If the embryo was dead, that egg was not considered in the calculations.

Determination of Neutralizing Antibody Titers of Vaccinated and Naturally Exposed Mink.—The 45 mink used in this experiment were divided into four groups. Group A, consisting of 12 mink, served as controls, and were not vaccinated. Group B, containing 13 mink, were vaccinated Nov. 28, 1950, with 0.5 cc. each of HG vaccine, prepared as described above.

The animals of group C, consisting of 10 mink, were similarly vaccinated Nov. 28, 1950. Group D, also containing 10 mink, was not vaccinated. Groups C and D were immediately placed in small wire cages in a small isolation room along with 23 ferrets which were naturally infected with distemper.

The mink in groups A (control) and B (vaccinated) remained healthy throughout the experiment. A representative number of mink from both groups were bled at various intervals up to Jan. 11, 1951, at which time all the mink were bled and the experiment terminated.

The mink in groups C (vaccinated) and D (nonvaccinated) were exposed to the infected ferrets from November 28 to January 9. During this period, 4 of the nonvaccinated mink exhibited a slight catarrhal exudate from the eyes and nose, suggestive of a mild distemper infection, for two to three weeks. These catarrhal signs subsided prior to the termination of the experiment. None of the 10 mink in the vaccinated group or the remaining 5 nonvaccinated animals displayed any signs of disease during the experiment. The mink were bled and destroyed on January 9. Twenty-two of the 23 ferrets succumbed to distemper prior to the termination of the experiment.

TABLE 2—Neutralizing Antibody Titers in Mink Serum Following Vaccination and/or Natural Exposure to Distemper

History	Date bled	Days after vaccination	No. serums tested	Serum n.d.$_{50}$*		
				min.	max.	mean
Group A						
Controls, nonvaccinated, nonexposed	11/30/50	....	3	0	0	0
	12/14/50	....	3	0	0	0
	1/12/51	....	11	0	0	0
Group B						
Vaccinated 11/28/50, nonexposed	11/30/50	2	3	0	0	0
	12/ 7/50	9	5	16	80	32
	12/15/50	17	5	100	3,200	630
	12/21/50	23	5	200	3,200	800
	12/28/50	30	5	2,000	20,000	4,000
	1/11/51	44	13	630	32,000	3,200
Group C						
Vaccinated 11/28/50, exposed to infected ferrets 42 days	1/ 9/51	42	10	1,000	10,000	3,200
Group D						
Nonvaccinated, exposed to infected ferrets 42 days	1/ 9/51	....	9	20	32,000	400

* Expressed as reciprocal of 50 per cent neutralizing dose dilution.

The serums collected from these four groups of mink were tested for neutralizing antibodies by the technique described above. The results of these tests are shown in table 2. It can be seen that the two groups of vaccinated mink (groups B and C) developed relatively high titers of neutralizing antibodies during the postvaccination period, and that exposure to infected ferrets during this period had no effect on the level of antibodies attained in group C.

None of the serums from the nonvaccinated control animals (group A) contained detectable amounts of neutralizing antibodies. However, all of the nonvaccinated mink in group D developed significant titers of antibodies as a result of their exposure to the infected ferrets, although only 4 of them showed evidence of a possible mild infection with the virus. Significantly, 1 of the mink in this group developed a titer equal to the highest titer recorded in the vaccinated groups.

The amount of virus used in the serum-virus mixtures varied from 300 to 3,000 i.d.$_{50}$ per inoculum. This wide variation undoubtedly influenced the resulting serum titers. In group B, where more than one sample was collected from each mink, all samples from the same animal were tested at the same time. Therefore, the relative titers at the various periods should be reasonably accurate. Furthermore, the mean titers on the various days indicate a consistent rise up to thirty days after vaccination, with a very slight drop by forty-two days.

Determination of Neutralizing Antibody Titers of Vaccinated Ferrets.—The purpose of this experiment was to determine the relative neutralizing antibody titers in ferret serum collected at various periods following vaccination with HG virus and, at the same time, to determine at what period following vaccination resistance to virulent virus developed.

In this experiment, ten groups of 3 ferrets each were vaccinated with the HG virus at various intervals from Nov. 13, 1951, to Dec. 12, 1951, each ferret receiving a single inoculation of 0.5 cc. of vaccine. All ferrets were bled, prior to vaccination, for control serum. On Dec. 10, 1951, all of the above ferrets plus 3 normal, nonvaccinated controls were bled to supply serum for neutralization tests, then immediately challenged with virulent distemper virus (Green's distemperoid).

The procedure and results of this trial have been previously reported [1] but will be briefly reviewed here. All ferrets vaccinated two or more days prior to challenge showed no signs of distemper with the exception of 1 animal. This animal (ferret 3 in group 1), which was vaccinated twenty-seven days prior to inoculation with virulent virus, died thirteen days following this challenge showing typical signs of the disease.

The remaining ferrets, *i.e.*, those which had been vaccinated simultaneously with challenge, or vaccinated two days after challenge, and the 3 control animals, all developed typical signs of distemper and succumbed or were destroyed when moribund.

The serums collected just prior to challenge, representing various intervals before and after vaccination, were tested for neutralizing antibodies. Table 3 shows the results of these tests. The fate of each ferret following challenge is also indicated in this table in order to compare the time of development of resistance to virulent virus with the time of appearance of detectable neutralizing antibodies.

Neutralizing antibodies were detected in all but one of the serums taken seven or more days after vaccination. Antibodies were not detected in the serums taken two and four days after vaccination, even though the ferrets were found to be resistent to virulent virus at that time. Neutralization was not demonstrated in any of the samples collected prior to vaccination.

The absence of neutralizing antibodies in the serum of ferret 3 in group 1 is difficult to explain, but the development of typical distemper and resulting death of this ferret following challenge provides an interesting correlation.

The amount of virus used in these tests ranged from 100 to 800 i.d.$_{50}$ per inoculum. Two series of tests, in which less than 100 i.d.$_{50}$ were used, were repeated.

DISCUSSION

A quantitative serum-virus neutralization test for distemper has been described in this paper. The quantity of virus used in the test must be controlled within narrow limits in order that multiple titrations of the same serum give consistent results. In our experience, this has been difficult to accomplish. Therefore, until the test is standardized, it is desirable to titrate all serum samples from 1 animal, or from a group of animals which are to be compared, at the same time in order to determine accurately the relative antibody titers. In our opinion, however, this variation does not nullify the value of the test.

The presence of low levels of neutralizing antibodies was readily determined.

Neutralizing substances were not detected in the serums of any animals not previously exposed to the virus. These results show that neutralization is indicative of previous contact with the distemper virus.

In the present study, one strain of egg-adapted virus was employed. Because of the possible occurrence of antigenically different strains of virus, simultaneous testing with several strains of egg-adapted virus is advisable.

In the ferret experiment, it was found that resistance to infection with virulent virus developed within two days following vaccination with the avirulent egg-adapted virus, whereas neutralizing antibodies were not detected until seven days following vaccination. The mechanism of this early resistance is not understood. Karzon and Bang,[8] working with Newcastle disease virus, found that circulating antibodies were first detectable three days following vaccination with inactivated virus but could not be detected until six days following an infectious inoculum. The appearance of circulating antibodies coincided with a decline of virus titers in the tissues, suggesting the possibility that antibodies formed prior to the sixth day were bound by the virus in the tissues. It is possible that a similar mechanism was operating in our experiment.

A satisfactory serum-virus neutralization test for distemper should prove to be a valuable tool in the study of this disease.

TABLE 3—Immunity Response of Ferrets to Distemper Vaccination

Group (No.)	Days after vacc. when bled and challenged	Ferret (No.)	Serum $n.d._{50}$* at time of challenge	Fate** of ferret after challenge with virulent distemper virus
11	Controls, not vacc.	1	0	D }
		2	0	K } 12 days—distemper
		3	0	K }
10	−2	1	0	K }
		2	0	K } 12 days—distemper
		3	0	K }
9	0	1	0	D }
		2	0	D } 14 days—distemper
		3	0	D }
8	2	1	0	NS
		2	0	NS
		3	0	NS
7	4	1	0	NS
		2	0	NS
		3	0	NS
6	7	1	TR+	NS
		2	TR	NS
		3	TR	NS
5	10	1	25	NS
		2	16	NS
		3	8	NS
4	13	1	40	NS
		2	25	D 21 days—undetermined
		3	4	NS
3	16	1	20	NS
		2	2	NS
		3	13	NS
2	20	1	130	NS
		2	130	NS
		3	630	NS
1	27	1	1000	NS
		2	1000	NS
		3	0	D 13 days—distemper

* Expressed as reciprocal of 50 per cent neutralizing dose dilution; ** K = killed when moribund, D = died; number of days after challenge and diagnosis are indicated; NS = no signs of illness in 31-day observation period; TR+ = Trace, < 2.

The possible uses for such a test include: (1) If it could be demonstrated that the level of distemper neutralizing antibodies is correlated with the degree of active resistance in the host or the degree of passive resistance conferred to the recipient of the immune serum, the neutralization test should serve as a valuable adjunct to animal protection tests in evaluating and standardizing distemper vaccines and therapeutic antiserums; (2) It is often difficult to differentiate, on either clinical or pathological bases, certain conditions which simulate distemper. The demonstration of a rising titer of antibodies during the disease would help to establish the correct diagnosis. Conversely, the absence of a rising titer might help to elucidate other clinical entities; (3) The existence of antigenically different strains of DV has been the subject of a lengthy controversy which has yet to be resolved. The neutralization test has been instrumental in identifying and studying the antigenic structure of various strains of the neurotropic viruses infecting man and should be equally adaptable to this type of study on the distemper virus; (4) The neutralization test should be of particular value in studying the ecology of distemper and determining the possible occurrence of unsuspected reservoirs of infection. Ott and coworkers,[11] in preliminary studies using the technique described above, found high titers of neutralizing antibodies in dogs with no history of vaccination or frank infection with the virus. This finding provides evidence of inapparent infections and suggests the occurrence of healthy carriers of the virus.

Although Savan and Brandly[13] and Mansi[10] have designed complement-fixation tests for distemper, this test has not been successful in our laboratory because of the difficulty of preparing satisfactory antigens and the anticomplementary activity of many dog serums.

Summary

A quantitative *in ovo* neutralization test for distemper, using the serial serum dilution-constant virus procedure, is described.

The test was applied to mink and ferret serums collected under various conditions of exposure to distemper virus.

In ferrets vaccinated with egg-adapted distemper virus, circulating antibodies were first detected seven days following vaccination whereas resistance to virulent virus developed within two days.

Rising titers of antibodies were demonstrated in mink and ferret serums from the seventh to the thirtieth day following vaccination.

Neutralizing antibodies were detected in all serums from a group of nonvaccinated mink that were exposed to distemper-infected ferrets.

Serums collected from animals not previously exposed to the virus exhibited no neutralizing ability.

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Lesions in Chicken Embryos Produced by Pleuropneumonia-Like Organisms from Chronic Respiratory Disease of Chickens and Infectious Sinusitis of Turkeys

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THE DIFFERENTIAL diagnosis of poultry diseases, particularly of the respiratory system, has become increasingly important. Most veterinary diagnostic laboratories and research institutions use the chicken embryo as a diagnostic aid in poultry virus and other respiratory disease conditions. Chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys have been thought to have an identical etiological agent. It is the purpose of this report to compare the pathological effects of nine chicken and four turkey strains of pleuropneumonia-like organisms (p.p.l.o.).

LITERATURE CITED

In 1935, Nelson [13] found coccobacilliform bodies in association with cases of fowl coryza. Gibbs [5] in the same year described a similar agent in chickens associated with coryza. Nelson [14-17] concluded in 1936 that these coccobacilliform bodies of 0.5 μ or less in diameter produced a respiratory infection in chickens by direct contact and had a longer incubation period than coryza caused by *Hemophilus gallinarum*. He found the organisms became grouped in clumps and might become large and compact, comprising innumerable units. In 1939, he cultured these bodies in tissue culture and in 1950 concluded these bacilliform bodies and p.p.l.o. were very similar. Delaplane and Stuart [3] in 1943 isolated an agent from hens. They reported it as a virus and succeeded in passing it in chicken embryos 35 times. This virus was capable of reproducing a low grade respiratory disease in old and young birds six to fourteen days post-inoculation. In 1948, Delaplane [2] reported 7-day-old embryos to be preferable for propagation of the agent. Gross lesions consisted of hemorrhage of the liver, kidneys, and brain, whitish circumscribed foci on the chorioallantoic membrane, and purulent inflammation of the joints. Delaplane [4] in 1950 stated that the chronic respiratory disease "virus" of chickens and the infectious sinusitis agent of turkeys were identical. Van Roekel, Olesiuk, and Peck [20] in 1952 described the clinical signs of CRD in chickens and reported the occurrence of this disease in 53 flocks. They also observed gross lesions in chicken embryos consisting of stunting, edema, congestion, hemorrhage, abscess (joints) as well as congestion, necrosis, and enlargement of kidneys and livers. In 1952, Olesiuk and Van Roekel [18] reported that they had been able to grow the organism in artificial broth mediums up to 29 serial passages and that it probably belonged to the p.p.l.o. group. Markham and Wong [12] reported in 1952 that they had successfully cultured p.p.l.o. (in 20 per cent horse serum and beef infusion broth) from yolk sac suspensions of chicken embryos inoculated with egg passage strains of CRD and turkey sinusitis agents. After 13 consecutive passages in artificial mediums, representing a 10^{20} total dilution, these agents induced typical swelling of turkey sinusitis when injected into the infraorbital sinus. Using Giemsa's stain, microörganisms similar to p.p.l.o. were demonstrated from yolk sac passage strains and field exudates.

Groupe *et al.*[6] in 1948 studied sinus exudate from turkeys which had been inoculated with the sinusitis agent grown in chicken embryos. Impression smears stained by Macchiavello's technique revealed coccoid bodies similar to those found in the lymphogranuloma-psittacosis group (Chalmydozoaceae). Hitchner [9] in 1949 described the pathology of infectious sinusitis of turkeys. Giemsa-stained smears revealed bodies suggestive of rickettsial organisms. Jungherr [10] in 1949 observed lymphogranuloma-like elementary bodies and cysts in yolk sacs infected with a high titer sinusitis agent. Chicks, 2 and 4 weeks of age inoculated parenterally and intracerebrally, developed small granulomas of the liver. Jungherr and Luginbuhl [11] reported in 1952 that chickens with CRD showed marked mononuclear infiltration in the trachea, lung, and air sacs as well as focal granulomas with giant cells in the air sac and pericardium. Grumbles *et al.*[7] in September, 1952, reported natural transmission of infectious sinusitis of turkeys to chickens in a series of three experiments.

MATERIALS AND METHODS

The eggs used for this experiment were obtained from a flock with a history of no respiratory disease.

The strains of pleuropneumonia-like organisms were obtained from three sources. Dr. Henry Van Roekel, University of Massachusetts, Amherst, provided seven strains; *viz.*, A, B, C, D, E, F, and

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G. Strains I, K, and J were received from Dr. Floyd Markham, Lederle Laboratories, Pearl River, N. Y. Three strains M, N, and L were obtained from Dr. B. S. Pomeroy, University of Minnesota, St. Paul. Table 1 indicates the history and characteristics of the strains studied. The strains were maintained in a deep freeze unit at a constant temperature of —20 C.

The test strain of the organism to be used was removed from the deep freeze and allowed to thaw at room temperature. The organism was diluted in nutrient broth and inoculated immediately into 7-day-old chicken embryos by the yolk sac route. The eggs were returned to the incubator and candled daily until hatching. Fourteen live, healthy, uninoculated controls were used each time a strain was studied.

The minimum lethal dose was determined to the nearest 50 per cent. Five eggs were used for each dilution. Embryos which died during the first twenty-four hours were cultured and if sterile were considered insignificant and due to traumatic causes.

A bacteriological loopful of yolk material from all embryos which died at 8 to 21 days of incubation was inoculated into a tube of thioglycollate (B.B.L. 135) to test for contamination. Three smears were made from the yolk stalk. The middle toe of all embryos 12 days old or older was measured. This measurement was made from the middle of the metatarsal joint to the tip of the toe. This was used as a criterion for dwarfing. Hamilton [8] has shown normal third toe measurements. Gross observations were made of the embryo, yolk sac, and chorioallantoic membrane (c.a.m.).

All embryos were fixed in 10 per cent formol-saline solution except those which were fixed in Zenker's, Petrunkewitch's, or Bouin's fluid for comparative purposes. All embryos inoculated (40 in titration) with strain A and D were examined histologically. In all other strains, embryos showing gross lesions and those which died prior to 21 days of incubation were sectioned. Saggital and cross sections were made of embryos dying before 14 days of incubation. The following organs from 12- to 21-day-old embryos were sectioned: heart, liver, leg or wing joint, lung, brain, kidney, and any other organ showing gross lesions.

Stains used routinely for tissue sections were hematoxylin and eosin and Giemsa. In addition, Unna's polychrome methylene blue stain was used for some sections. Giemsa's, Macchiavello's, Victoria Blue R, and Castaneda's stains were used for yolk stalk smears. Both Wilder's and Bodian's stains were used for the detection of reticulum fibers.

Twenty healthy mice were inoculated intraperitoneally. Ten received an inoculum composed of nine chicken p.p.l.o. strains, 10 received an inoculum consisting of turkey sinusitis strains.

RESULTS

GROSS PATHOLOGY

The embryos which died between 8 to 14 days of incubation usually showed extensive hemorrhage and edema. Transudate oozed from all sides of many embryos when observed on a glass plate. Ecchymotic hemorrhages were noticed frequently in the skin of the head, neck, and ventral thoracic region. Figure 1 demonstrates a 14-day-old infected embryo with a thickened, opaque c.a.m., sparse feathering, generalized edema, and surrounded by transudate in comparison to an uninoculated control.

TABLE 1—History and Characteristics of Strains of Pleuropneumonia-Like Organisms Studied

Strain	Source	Species origin	No. of passages	Minimum lethal dose (%)	Embryo mortality pattern deaths at days incubation	Special features
	Van Roekel	chicken	4	44.4 at 10^{-3} dilution	9, 20.	Few lesions.
B	Van Roekel	chicken	1	50 at 10^{-1} dilution	17, 20.	Low titer; few lesions.
C	Van Roekel	chicken	2	50 at 10^{-4} dilution	9, 11, 13, 16, 19, 20.	High titer; many lesions.
D	Van Roekel	chicken	21	43.7 at 10^{-3} dilution	14, 15, 17, 18, 20.	Many lesions.
E	Van Roekel	chicken	1	44.4 at 10^{-1} dilution	16, 20.	Low pathogenicity for embryos.
F	Van Roekel	chicken	8	50 at 10^{-1} dilution	14, 16, 20.	Low titer.
I	Markham No. 1136	chicken	82	45 at 10^{-3} dilution	10, 17, 19.	Strain producing granulomas.
K	Markham (W.T.)	chicken	41	50 at 10^{-4} dilution	9, 10, 13, 15, 16, 19.	Granulomas produced.
N	Pomeroy (Y)	chicken	....	66 at 10^{-1} dilution	9, 10, 11, 17, 19, 20.	Low pathogenicity for embryos.
G	Van Roekel	turkey	9	52.1 at 10^{-4} dilution	9, 10, 11, 12, 13, 14, 15, 19, 20.	High titer.
J	Markham No. 2037	turkey	88	45 at 10^{-3} dilution	9, 10, 12, 15, 17, 20.	Many lesions.
L	Pomeroy (B)	turkey	....	50 at 10^{-2} dilution	10, 11, 12, 16, 18.	No deaths 19 or 20 days.
M	Pomeroy (I)	turkey	....	45 at 10^{-5} dilution	9, 10, 12, 13, 14, 17, 18, 19, 20.	Very pathogenic for embryos.

Embryos which died between 14 to 21 days of incubation presented hemorrhage and edema. The tibiofemoral articulations were white in contrast to the remainder of the bloodstained legs. Edema was prevalent in the head, dorsal neck, sublingual, as well as in the lumbar and thoracic vertebral regions. Many embryos were definitely dwarfed (fig. 2) as shown by the following examples of middle toe measurements: 0.9 cm. in a 13-day-embryo (normal 0.98 cm.), 1.5 cm. in an 18-day-embryo (normal 2.04), and 1.6 cm. in a 20-day embryo (normal 2.14). The method of third toe measurement is shown in figure 3. Chorioallantoic membranes were thickened, opaque, dry, and closely adherent to the embryo. A few embryos at 20 days' incubation appeared even larger than usual (fig. 4). Much of this apparent body enlargement and shortness of the legs was attributable to severe generalized edema which was extremely prominent around the head, neck, and abdomen. The leg and wing joints were puffy, white and fluid poured out when they were sectioned. Edematous wing joints often contained minute bubbles of white material. This could have been mistaken for pus but microscopically was homogenous acellular material rather than pus. Many livers were enlarged and hyperemic while others had a granular surface and contained small white necrotic areas (fig. 5). Pleuritis and renal hemorrhage were not uncommon.

HISTOPATHOLOGY

Embryos 9 to 14 Days of Age.—Transverse sections from embryos up to 14 days of age showed myocardial hemorrhages and passive hyperemia. The skin and muscles in the region of the vertebrae were edematous as evidenced by pink homogenous material in the interstices. The spinal cord showed areas of disintegration and infiltration of lymphocytes. These cells had also infiltrated into the splanchnic ganglia. There were hemorrhages and lymphocytic foci throughout the lungs. The livers presented general passive congestion and hepatic cell necrosis around many central veins as evidenced by pyknosis, karyorrhexis, and karyolysis. The sinusoids contained many lymphocytes. The kidneys showed mild to severe interstitial hemorrhage. More lymphocytes than "normal"

Fig. 1—(Left) Normal 14-day-old embryo and chorioallantoic membrane; (right) edema, hemorrhage, and thickened opaque chorioallantoic membrane of an infected 14-day-old embryo.

were seen in the bone marrow. The embryonic brain showed diffuse edema and gliosis. The distribution of lesions in the infected embryos is summarized in table 1.

Embryos Between 15 and 21 Days of Age.—Embryos dying between 15 to 21 days of incubation presented myocarditis characterized by hyperemia, edema, and lymphocytic infiltration throughout the myocardium. Endocarditis, fibrinous pericarditis with necrosis, and epicarditis were observed (fig. 6). Liver lesions were characterized by focal necrobiosis, minute to large foci of lymphocytes, parenchymatous degeneration, and hyperemia at the periphery of the organ (fig. 7). Periphlebitis involved the central veins and, to a lesser extent, the portal vessels. Heterophils infiltrated the perivascular spaces and walls of the veins. A chronic peritonitis was evidenced by heterophil infiltration and fibrosis of the liver capsule. Hemorrhage filled the tertiary bronchi and alveoli. Edema (fig. 8) occurred frequently in the lungs. Periarteriolitis, arteritis, and periarteritis were frequent lesions of the pul-

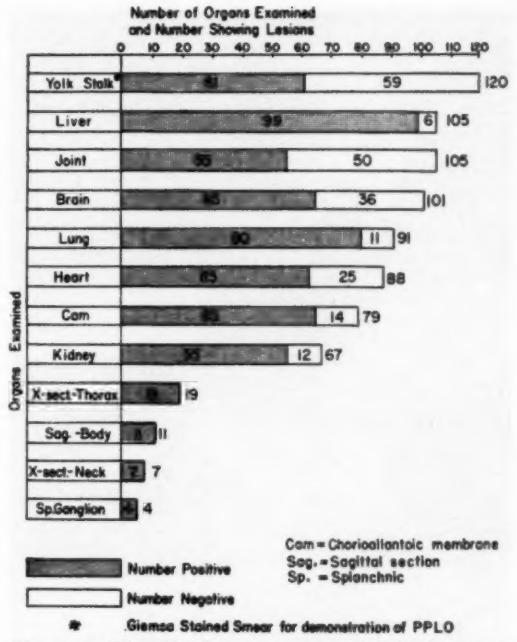

Chart 1—Distribution of lesions in chicken embryos infected with pleuropneumonia-like organisms.

Fig. 2—(Left) Normal 20-day-old embryo; (right) dwarfed infected 20-day-old embryo.

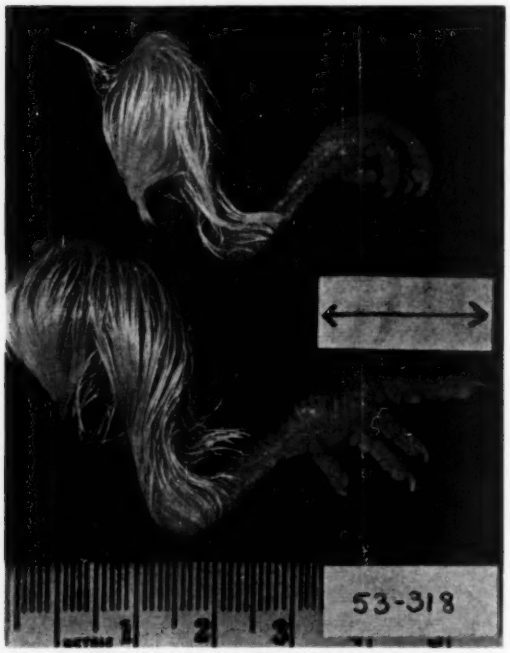

Fig. 3—(Top) Leg of 20-day-old dwarfed embryo; (bottom) leg of 20-day-old normal embryo with arrow indicating third toe measurement.

monary vessels. The perivascular spaces, adventitia, and tunica media of most arterioles and arteries were infiltrated with heterophils (fig. 9). The spinal cord, particularly in the region of the central canal and gray matter, was infiltrated with lymphocytes. The dura mater was thickened and infiltrated with lymphocytes. There was neuronal degeneration, edema, and diffuse gliosis of the brain. Thrombosis of many cerebral vessels was observed. The kidneys presented the same focal inflammation as was seen in the livers. Hemorrhage was severe and the renal tubular epithelium showed fatty and albuminous degeneration. Occasionally, there was necrosis in the joint cavities. Von Kossa's stain did not reveal any calcification in these areas of necrosis. The periarticular tissues contained lymphocytic foci, necrosis, hemorrhage, and discontinuity of many muscle fibers (fig. 10). Fibroplasia was evidenced by Masson's trichrome stain. The bone marrow showed masses of lymphocytes instead of the normal predominating granulocytic leukocytes. Extensive edema was noted in the leg and wing joints of some

Fig. 4—(Left) Normal 20-day-old embryo; (right) generalized edema, particularly of the neck and abdomen of a 20-day-old infected embryo.

surviving embryos. Cross sections of the anterior cervical region showed subcutaneous hemorrhages, separation of the muscle bundles, and swelling of myofibrils. Muscle fibers located at the periphery of muscle bundles were frequently hyalinized (fig. 11). The chorioallantoic membranes showed hyperplasia of the ectodermal layer, necrosis, hyperemia, and hemorrhage. Giemsa-stained sections often revealed several large, round, pale blue epithelial cells filled with eight to 12 round, discrete, dark, purple-staining organisms 0.5 to 0.9 μ in size (fig. 12). These were considered to be pleuropneumonia-like organisms.

Strain Differences.—The c.a.m. of embryos infected with strain C were opaque, thickened, and adherent to the embryo. White plaques measuring 0.5 mm. to 2 mm. in diameter were scattered over the membrane.

The most characteristic lesions produced by strain E were cerebral hyperemia and hepatitis.

Strain N produced severe dwarfing in numerous embryos and hydropericardium in one instance. Kidney sections showed scattered large glomeruli filled with lymphocytes. Some of the affected glomeruli were necrotic and calcified.

Chicken strain I produced dwarfing of embryos; hyperemia and necrosis were frequent hepatic lesions. The most significant finding was that of circumscribed yellow to white raised nodules, varying in size from 1 to 4 mm. in diameter, found under the skin of the tibial and femoral portions of the legs, on the hock, toe, wing joints, and in the gizzard (fig. 13). Microscopically, the nodules were composed of a necrotic center surrounded by epithelioid cells which had coalesced to form giant cells containing 2 to 27 nuclei (fig. 14, 15). Neocapillaries and hemorrhage were seen throughout the granulomas.

Although in chicken strain K no subcutaneous granulomas were seen grossly, microscopic study of the subcutaneous periarticular tissues revealed diffuse granulomatous inflammation. Giant cells were not evident.

Using Giemsa's stain, p.p.l.o. were identified in 28.3 per cent of the yolk stalk smears examined. Pleuropneumonia-like organisms were also noted in 11.1 per cent of the c.a.m. examined.

DISCUSSION

A study was made of nine strains of an organism capable of causing chronic respiratory disease in chickens and 4 strains of a similar organism producing infectious sinusitis of turkeys. These p.p.l.o. when injected into embryonating chicken eggs produced an erratic death pattern extending over a two-week period.

The gross lesions as reported by Delaplane,[1,3] Hitchener,[9] and Van Roekel[20] in chicken embryos from chicken and turkey strains have been hemorrhage and necrosis of the liver and kidneys, pneumonia, edema, abscessation of joints, dwarfing, and various c.a.m. lesions. In the present study, similar gross lesions were noted, except that pneumonia was infrequent and joint abscessation was absent. Edema of the head, neck, and joints, and hemorrhagic thickened, opaque c.a.m. occurred more frequently in this study than has been reported previously.

Fig. 5—(Top) Normal liver; (bottom) yellow necrotic foci.

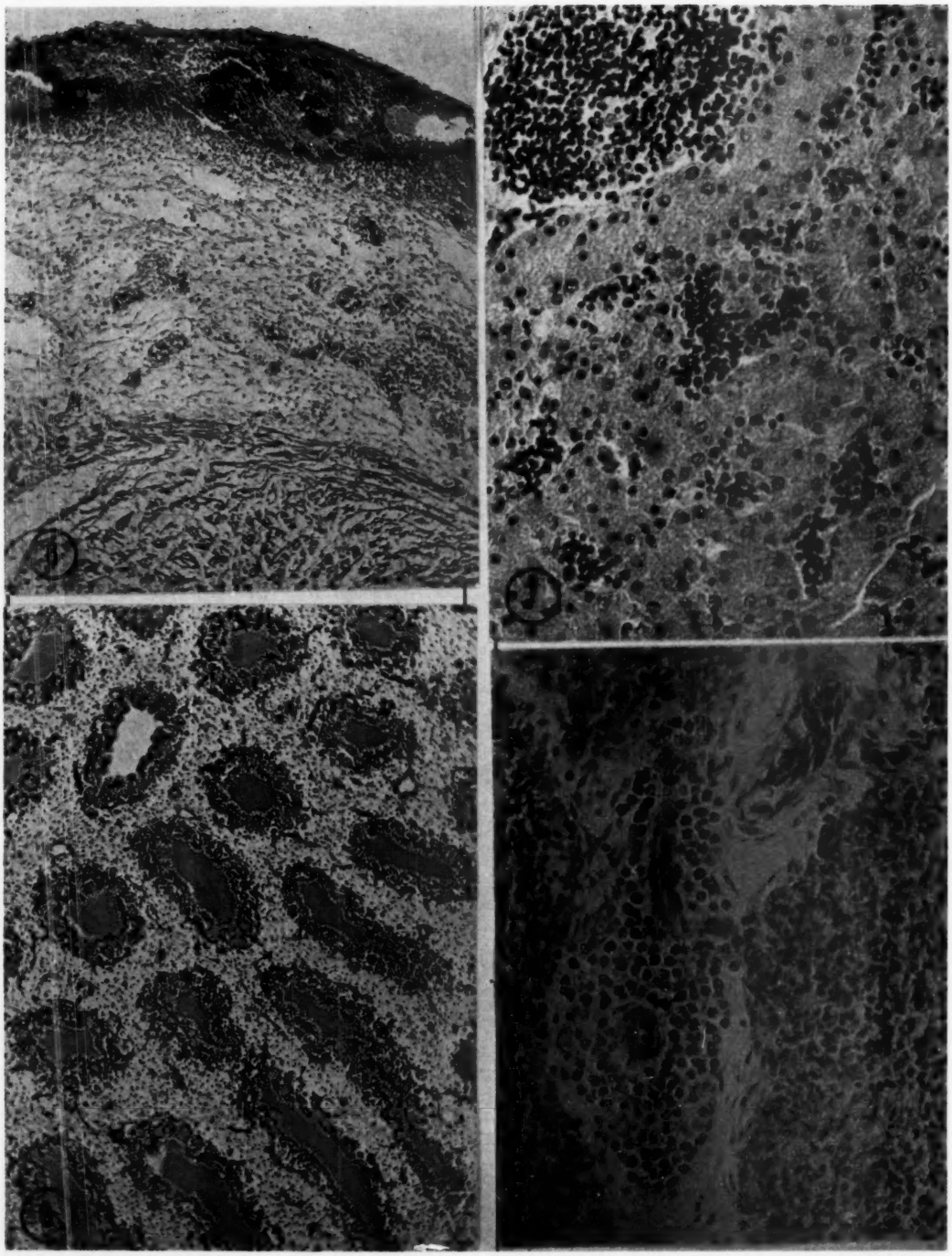

Fig. 6—Section of heart showing necrosis of pericardium, pericarditis, and epicarditis. Hematoxylin and eosin. x 85.

Fig. 7—Section of liver showing central veins, sinusoids, and focal hepatic lesions containing lymphocytes. Hematoxylin and eosin. x 420.

Fig. 8—Section of lung showing edema. All tertiary bronchi except one are filled with transudate. Hematoxylin and eosin. x 95.

Fig. 9—Section of lung showing arteritis and periarteritis. Heterophils have infiltrated adventitia and perivascular tissue of an arteriole. Pulmonary tissue contains many lymphocytes. Giemsa. x 400.

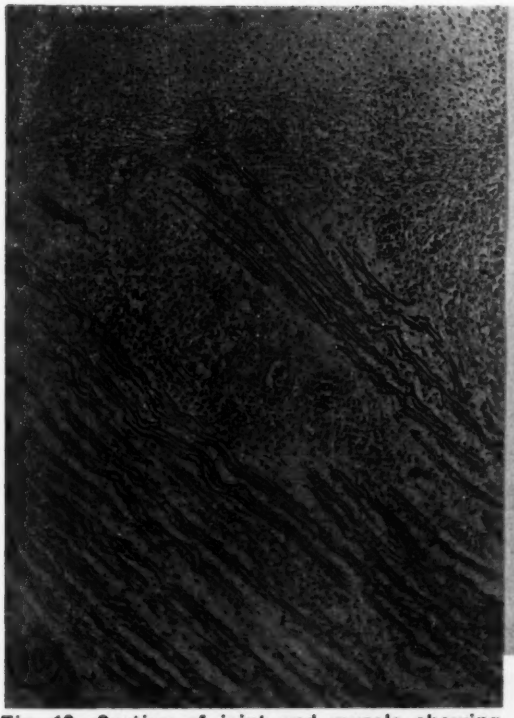

Fig. 10—Section of joint and muscle showing myositis and disruption of muscle fibers. Hematoxylin and eosin. x 100.

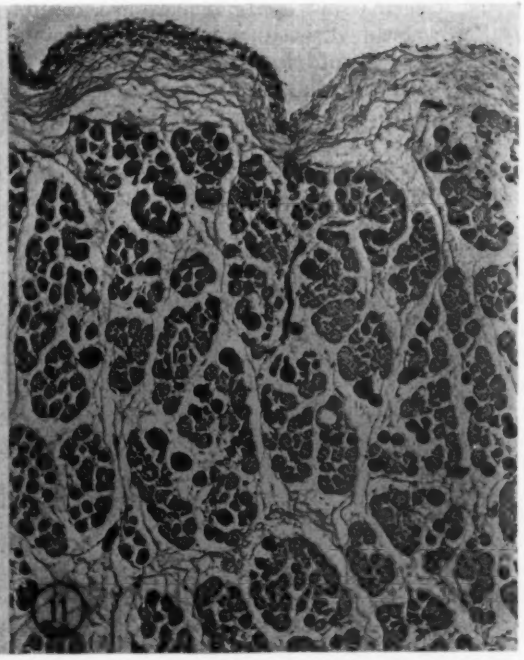

Fig. 11—Section of neck muscle showing hemorrhage and edema of the perimysium and muscle bundles separated by edema. Many muscle fibers hyalinized. Hematoxylin and eosin. x 80.

Hyperemia, hemorrhage, and edema were microscopic lesions of greatest frequency in embryos dying up to seven days postinoculation. These changes were prevalent in all organs including the c.a.m. Pleuropneumonia-like organisms were seen in yolk stalk smears and in tissue sections of the c.a.m. This would indicate that death was due to vascular changes accompanied by transudation, extravasation, and interference with transfer through the c.a.m.

Microscopically, embryos dying seven to fourteen days after inoculation, presented in addition to vascular changes degeneration, periarteritis of the pulmonary vessels, periphlebitis of the liver vessels, and lymphocytic foci in many organs. Most strains produced subacute infection. In three strains, granulomatous inflammation was produced which was evidence of a more chronic condition. Death may have been the result of complete destruction of some vital organ; *viz.*, c.a.m., liver, heart, brain, or kidney.

Although, grossly, CRD infection in chicken embryos could be confused with infection caused by infectious bronchitis

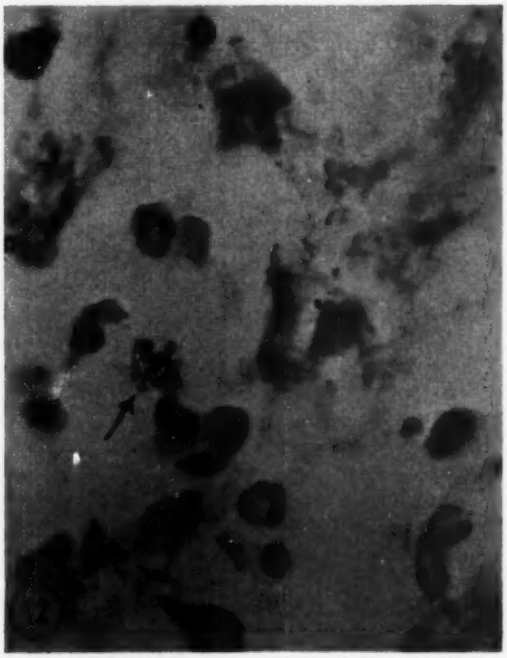

Fig. 12—Section of chorioallantoic membrane with pleuropneumonia-like organism in mass of necrotic debris. Note the centrally located nine dark-staining round bodies surrounded by a thin cell membrane. Giemsa. x 1350.

virus, it seems that acute infections caused by Newcastle disease virus or bacterial organisms *(Streptobacillus moniliformis)* could be differentiated microscopically.

Microscopically, infectious bronchitis virus does not produce lymphocytic infiltration or granulomas which were seen in CRD. Organisms causing bacterial infections can be demonstrated in Giemsa-stained tissues. In this study, no lesions were observed in uninoculated controls. Sabin and Warren [19] were able regularly to produce progressive proliferative arthritis two to four months after intravenous injection of a fresh artificial culture of the organism. We were unable to produce progressive proliferative arthritis two to four months after intravenous injection of a fresh artificial culture of the organism. We were unable to produce any significant lesions in mice within two months after intraperitoneal injections with chicken or turkey strains from yolk sac or allantoic fluid material.

SUMMARY

A specific pattern of embryo mortality could not be associated with the strain (chicken or turkey) used, or the number of times the organism had been passed artificially.

The minimum lethal dose of the organism (in chicken embryos) was higher after the pleuropneumonia-like organism (p.p.l.o.) was apparently adapted (numerous passages) to the embryo and lower in first or early passage strains.

Hemorrhage and edema were the most common lesions produced during the first week after inoculation. In descending order of frequency, gross lesions seen between fourteen and twenty-one days of incubation were dwarfing, hyperemia of livers, opaque and thickened chorioallantoic membranes, generalized edema and hemorrhage, necrotic foci in the livers, sparse feathering, edema of the head, neck and joints, and kidney hemorrhage.

Fig. 13 — A 19-day-old embryo with granulomas of legs, wing, and gizzard.

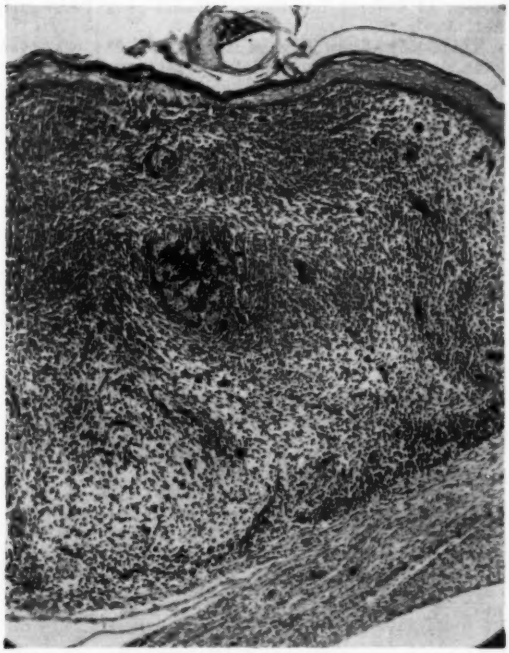

Fig. 14 — Periarticular granuloma of tarsal joint. Fibroblasts, heterophils, and lymphocytes at bottom close to muscle tissue. Center composed of necrotic debris surrounded by epithelioid giant cells. Neocapillaries, periphlebitis, and periarteritis. Hematoxylin and eosin. x 85.

The microscopic picture indicated that early mortality was of an acute nature whereas chronic to granulomatous changes occurred late in incubation.

Hyperemia, hemorrhage, periarteritis, arteritis, and periphlebitis were vascular changes of frequent occurrence in all organs. Periarteritis occurred in more than 51 per cent of the lungs. Arteritis was less frequent than periarteritis and was found in the lungs and joints. Periphlebitis was observed in 28 per cent of the livers sectioned.

Lymphocytic foci were common in many organs but most frequent in the liver where it occurred in 53.3 per cent of those examined. Heart sections showed pericarditis, myocarditis, and endocarditis.

Giemsa's stain for impression smears was concluded to be superior to Victoria blue, Macchiavello's, Unna's polychrome methylene blue, and Castaneda's stains for identifying the organism. Zenker's fixation followed by hematoxylin and eosin stain was preferred for architectural detail and cell morphology.

Twenty mice each receiving an inoculum of nine chicken strains of p.p.l.o. and 20 mice receiving a single inoculation of four turkey strains of p.p.l.o. did not develop any significant lesions in eight weeks.

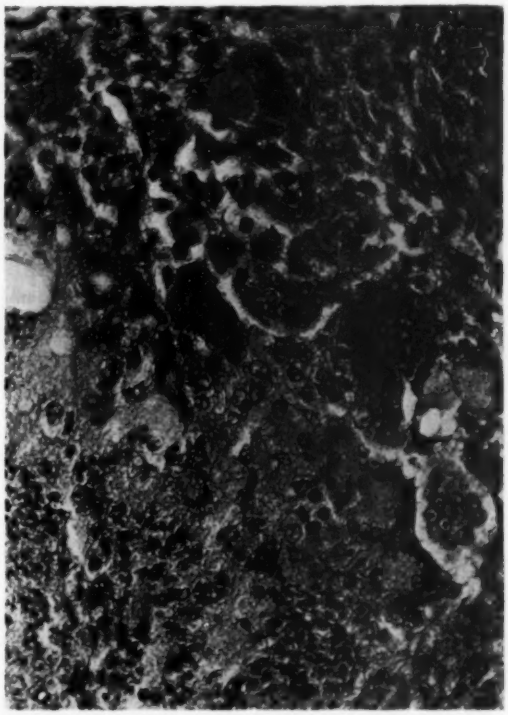

Fig. 15—Section of gizzard showing granuloma consisting of necrosis and masses of epithelioid and reticulum cells. Epithelioid giant cells are located at the periphery of a necrotic center. Hematoxylin and eosin. x 450.

Results of the pathological study indicated that: (1) Embryos which died two to seven days after inoculation showed hyperemia, hemorrhage, and edema caused by p.p.l.o. infection; (2) vascular changes and inflammatory foci progressing to granulomatous nodules were characteristic lesions of embryos dying eight to fourteen days postinoculation; (3) chronic lesions were more frequent in embryos inoculated with adapted strains of p.p.l.o.; (4) minor variations in strains were observed on the basis of gross and microscopic lesions and the course of the disease in embryos; (5) no significant differences between p.p.l.o. of chicken and turkey origin were detected.

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The Presence of Chronic Respiratory Disease in Pipped Eggs

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CHRONIC respiratory disease was first reported by Delaplane and Stuart [1] in 1943. It has spread rapidly and widely until today it is the most important malady affecting the broiler industry. During the winter of 1951-1952, it became enzoötic in the "Delmarva" area and this was associated with a pronounced increase in severity and mortality. In addition to the mortality, there was a marked decrease in feed conversion and quality of broilers produced. These factors cost the Delmarva poultrymen about $20,000,000 over an eight-month period.

Delaplane and Stuart,[1] in their initial report, suggested that the agent was viral-like in its characteristics. Nelson [5-9] was the first to report the finding of coccobacilliform bodies in the exudate of infection in the upper respiratory tract of chickens. Groupe,[3, 4] Eaton,[2] Van Roekel,[10] and others have added additional evidence to support the theory that a pleuropneumonia-like organism (p.p.l.o.) is at least a part of the etiology of chronic respiratory disease (C.R.D.). Van Roekel[10] reported the presence of tracheal pneumonic and air sac lesions typical of C.R.D. in embryos and chicks obtained from commercial hatcheries. He also identified the C.R.D. agent in these embryos by embryo and bird inoculation. However, many facts regarding the etiological agent or agents of this syndrome need clarification. The p.p.l.o. has been cultured on artificial mediums and can be successfully stained by Giemsa's and Michavello's method. The recent reports of an ornithosis-like agent in cases of respiratory disease of chickens add a disturbing element to the picture because of the possibility of transmission to and from man.

MATERIALS AND METHODS

From July, 1952, to May, 1953, this laboratory has received 70 samples of pipped live or dead embryos. Each sample consisted of two dozen pipped eggs originating from one flock and taken from the trays at hatching time. The lots include samples from four hatcheries. A record of the source of these eggs, as well as the chick placement from each lot, was recorded in order to check the health of each. A pool of tissue samples (lung, syrinx, trachea) from six eggs was made and an inoculum prepared by tituration with sterile sand and broth. In addition, one pooled inoculum was made from each two-dozen egg sample for inoculation into live birds. Thus, from each two-dozen egg sample, four inoculums were prepared for embryo passage and one pooled inoculum to be tested by injection into apparently healthy birds.

Embryo Passages.—For each milliliter of inoculum to be used in embryo passage, 10,000 units of penicillin were added. Four, 7-day-old embryos were used to test each inoculum and 0.2 ml. of the sample was injected into the yolk sac. Thus, 16 eggs were used for each egg lot; four, for each inoculum prepared. These eggs were candled daily to check mortality. All embryos which died during the first forty-eight hours were discarded. An embryo passage was considered positive if it showed the typical irregular mortality pattern and/or the presence of the typical lesions which characterize this disease. For each inoculum, four embryo passages were run in order to ascertain a positive or negative sample. When mortality occurred, the egg was chilled and then examined. Chorioallantoic fluid was harvested for future passage. After each harvest, this fluid was tested for its hemagglutination activity, as well as a bacteriological test on blood agar for sterility. If mortality did not occur, the eggs were removed from incubation at the eighteenth or nineteenth day and handled as mentioned above.

Live Bird Inoculation.—The pooled sample of four inoculums from each egg lot was tested by injection of live birds. No antibiotic was added to these inoculums. One chicken and 1 turkey, previously immunized against Newcastle disease by the use of killed virus, were used in these tests. Each bird received 0.5 ml. into the infraorbital sinus and 0.5 ml., intratracheally. These birds were held in isolation for twenty-one days. If symptoms of a respiratory disease occurred during this time, the birds were destroyed; or, if no symptoms occurred, they were destroyed on the twenty-first day. Serum from each bird was tested for hemagglutination-inhibition activity with Newcastle virus. Each bird was autopsied and the gross pathology noted.

The parent flocks represented by each egg lot sample were repeatedly visited to check the possible appearance of a respiratory disease. Likewise, all chick lots were checked during the growing period. In several cases, where respiratory disease occurred in these flocks, typically affected birds were sent to this laboratory for study.

RESULTS

The results of the tests, which were made on the 70 pipped egg samples, are

Published as miscellaneous paper No. 176 with the approval of the director of the Delaware Agricultural Experiment Station, Newark.

Grateful acknowledgment is made of the technical assistance rendered by Mr. Karl Seeger, Dr. Eugene Gill, Dr. Jaroslaw Geleta, Miss Barbara Jacobsen, and Mr. David Francis.

summarized in table 1. Twenty-nine samples were positive to embryo inoculation tests only, 21 samples were positive to embryo and live bird inoculation tests and 20

TABLE 1—Results of Tests on Pipped Egg Samples by Egg Lot

Source	Negative	Positive embryo passage	Positive embryo and live bird passage	Total
A	14	18	17	49
B	4	9	3	16
C	1	1	1	3
D	1	1	0	2
	20 (28 %)	29 (42 %)	21 (30 %)	70

samples were negative. There were no pipped egg samples which were positive to live bird inoculation but negative to embryo passage. These egg samples represent 39 parent flocks (table 2). Of these, 15 were reported to have shown symptoms of a respiratory disease during the experiment.

Eighteen broiler flocks representing corresponding pipped egg samples showed definite signs of a respiratory disease at some time during the growing period. There did not appear to be a connection between any of the following factors: (1) presence of respiratory disease in the parent flock, (2) presence of disease in the broiler flocks hatched from these parent flocks, (3) the presence of a positive laboratory test for the agent of chronic respiratory disease in pipped eggs representing these flocks.

One particular parent flock came under rather close observation because of a severe outbreak of chronic respiratory disease during the experiment. The disease was first recognized on Oct. 15, 1952. The symptoms observed were coughing, sneezing, a nasal discharge, and respiratory râles. Many birds showed swollen sinuses and some were observed to have an edema of the head, including the wattles. The birds became listless and refused to eat. The egg production rapidly declined to 10 per cent of its previous level. There was no apparent injury to shell texture or color. The overall mortality, including numerous culls, was 20 per cent in the flock of 12,000. The disease began at one end of the house and slowly progressed throughout the flock. Typically affected birds were submitted to this laboratory for examination. The pathology noted was similar to that which characterizes chronic respiratory disease. Briefly, there was a rhinitis and a sinusitis with the production of a thick, mucous exudate. An inflammatory condition was present in the lungs and bronchi, frequently with the production of a caseous exudate. The air sacs were thickened in many birds and were almost filled with a hard yellow exudate. In some birds, the liver was covered by a thick grey membrane. The pericardial sac was thickened and covered with an exudate in some cases.

Cultures of the lungs and air sacs of these birds showed the presence of staphylococci, streptococci, and many of the coli-aerogenes group of bacteria. Specific attention was given to the possible presence of *Salmonella gallinarum*, but this organism was not identified in any of the cultures prepared from these birds.

Several samples of typically affected birds were submitted for study and, from these, inoculums were prepared for embryo passages in the usual manner. Each inoculum was positive for a pleuropneumonia-like organism.

The disease lasted for eight weeks. At this time the flock has apparently recovered and resumed its normal feed consumption and egg production. Egg shell texture and color were apparently normal. No respiratory disease has occurred in this flock since.

When this severe outbreak occurred, it was decided to test a series of egg samples from this flock. It was fortunate that the laboratory had already tested a number of

TABLE 2—Results of Pipped Egg Tests by Parent Flocks Represented in Samples

Source	Disease in parent flock		Disease in broiler flocks		Tests on pipped eggs			
					Positive		Negative	Total
	Yes	No	Yes	No	Embryo only	Embryo and live bird		
A	14	2	10	39	18	18	19	55
B	1	17	8	10	11	3	4	18
C	0	3	0	3*	1	1	1	3
D	0	2	0	2*	1	0	1	2
Totals	15	24	18	54	31	22	25	78

Since some egg samples tested in the laboratory contained eggs from more than one source, the totals do not correspond.

* At autopsy and at time of slaughter, birds from these flocks showed lesions typical of chronic respiratory disease.

egg samples previous to the outbreak. Table 3 shows the results of these tests. It is significant that positive embryo passages occurred before, as well as sometime following, the natural outbreak of C.R.D. At the present time, May 19, 1953, the flock is in apparently good health and giving satisfactory results.

TABLE 3—Results of Tests on Pipped Egg Samples from One Flock Made Before, During, and Following a Natural Outbreak of Chronic Respiratory Disease

Egg sample No.	Date	Respiratory Disease: Parent flock	Respiratory Disease: Broiler flock	Passage: Egg	Passage: Bird
1	9/22/52	Negative	Negative	+	N
2	10/6	Negative	Negative	+	N
3	10/13	Negative	Positive	+	N
4	10/20	Became ill 10/15	Positive	+	+
5	10/26	Disease	Positive	+	+
6	11/3	Disease	Positive	+	N
7	12/16	Recovering	Negative	+	+
8	1/19/53	Negative	Negative	+	+
9	2/9	Negative	Negative	+	N
10	3/2	Negative	Negative	+	N
11	3/16	Negative	Negative	+	N
12	4/13	Negative	Negative	N	N

N—negative.

DISCUSSION

The presence of the causative agent of C.R.D. in 72 per cent of the egg lots tested may appear to be a high percentage. However, it should be noted here that these egg samples, in many cases, were taken from large incubators in which eggs from various sources were being hatched under commercial conditions. Also, these egg samples were in part taken from parent flocks which were having some respiratory distress. Taking the results of the embryo passage tests, it was not possible to correlate them with the presence of disease in the corresponding broiler flocks. In many cases it was observed that if the broiler flock came under some undo stress such as coccidiosis, bronchitis, etc., then C.R.D. would appear.

During the winter it became evident that these inoculums should also be tested for the presence of the ornithosis agent. At the present time, we are preparing to test all our cultures by checking their pathogenicity for mice.

One other factor should be mentioned relative to the pathology produced in the inoculated embryos. In 17 of the positive tests, there was noted definite curling of the embryos much like that seen in infectious bronchitis. Further study of these particular cultures will be made in the future in order to ascertain the possible presence or absence of bronchitis virus.

SUMMARY

During this experiment 70 lots of two-dozen pipped live or dead embryos were tested. Of these, 29 were positive to embryo passage, 21 were positive to both embryo passage and inoculation into chickens and turkeys, and 20 were negative. No definite correlation could be made between the laboratory tests and the health of the parent flocks, nor between these two and the health of corresponding broiler flocks.

A series of egg samples from one particular parent flock was tested, which contained egg samples taken before, during, and following a naturally occurring severe outbreak of C.R.D. There did not appear to be any direct correlation between the appearance of the disease in the flock and the presence of a positive embryo passage since the latter was positive for some time before and after the outbreak.

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A Testing Service for the Control of *Salmonella Typhimurium* Infection in Turkeys

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BACTERIOLOGICAL studies conducted during the past two decades have shown that many species of the genus Salmonella, other than *Salmonella pullorum*, may act as etiological agents of disease in turkeys. The resultant mortality varies markedly, a variation apparently influenced by shipping conditions, hatchery and brooder management, and species or strain of Salmonella involved.

A review of data on salmonellosis accumulated in the animal pathology laboratories of the California State Department of Agriculture showed that *Salmonella typhimurium* was more frequently incriminated as a cause of loss in turkey poults than any other species of Salmonella. All Salmonella spp., other than *S. pullorum*, were tentatively identified in these laboratories; the species were subsequently identified in the typing center at the University of Kentucky or in the laboratory of the California State Department of Public Health.

Hinshaw and McNeil [1] reported in 1943 on the use of an agglutination test as part of a procedure aimed at the control of *S. typhimurium* infection. Their report was based on trials wherein close field supervision was offered to members of three organizations in one turkey producing area of California.

Following requests from members of the turkey industry for assistance, the Bureau of Livestock Disease Control, California State Department of Agriculture, initiated a *S. typhimurium* detection service. The scope of the service was limited in 1949, but in 1950 it was made available on a state-wide basis. The two significant features of the service were that it did not require compliance with recommendations pertaining to flock as well as hatchery management, and participation was voluntary. This report describes the results of three years of operation of the testing service.

The limitations of such a service were recognized at the outset; however, the adoption of an official program at the time seemed premature. In lieu of a program with rigid controls, an attempt was made, through the dissemination of information concerning *S. typhimurium* infection, to impress members of the turkey industry with the importance of the field measures and to further emphasize that the test was primarily a detection procedure. A few of the more important features were as follows:

1) All replacement stock should be from eggs of flocks negative to the test for *S. typhimurium*.

2) Adherence to hatchery fumigation and other principles of sanitation should be followed.

3) Provision of separate hatching facilities for eggs from flocks tested and shown to be negative to the *S. typhimurium* test should be maintained.

4) There should be laboratory examination of poult specimens from broods showing symptoms of disease.

5) Measures for controlling rodent and other wildlife should be adopted, particularly in brooding and feeding areas.

A fee of 9 cents per test was established as a laboratory charge. Flock owners were requested to refrain from advertising test results. Blood was obtained for the test through the cooperation of the Poultry Improvement Advisory Board whose inspectors increased the quantity of blood

From the Bureau of Livestock Disease Control, California State Department of Agriculture, Sacramento.

Appreciation is extended to Dr. Arthur G. Boyd, chief, Division of Animal Industry, California State Department of Agriculture, and Dr. A. K. Carr, formerly administrator of the Division, now retired, for their guidance in the formulation of the testing service; and to Drs. W. H. Armstrong, Stanton L. Jamison, and W. W. Worcester for much of the data on isolations from reactors.

The authors are indebted to Dr. Ethel B. McNeil for the cultures used in antigen production and for many helpful suggestions; and to Dr. P. R. Edwards, U. S. Public Health Service, and Dr. Alcor Browne, California State Department of Public Health, for their assistance in serological typing of cultures.

The coöperation of Dr. R. A. Mueller, manager, California Poultry Improvement Advisory Board, in providing sufficient blood for both the *S. pullorum* and *S. typhimurium* tests is acknowledged.

TABLE 1—Summary of Agglutination Testing Results, Group 1

	Noninfected ranches				Infected ranches					
Season	No. ranches	No. birds	No. reactors	Reactors (%)	No. ranches	No. birds	No. reactors	Reactors (%)	Total No. ranches	Total No. birds
1949										
1950	1	2,752	1*	0.03	6	15,339	201	1.3	7	18,091
1950										
1951	2	7,576	52*	0.7	4	9,447	416	4.4	6	17,023
1951										
1952	3	4,532	0	....	2	9,482	270	2.8	5	14,014

* *Salmonella typhimurium* was not recovered from reactors.

collected from each bird over that required for the official pullorum test. The collection of blood in the event retests were necessary was the responsibility of the flock owners.

METHODS

Antigen Production.—The procedures used in antigen production, except for minor differences, were the same as those used by McNeil and Hinshaw.[2]

In the preparation of the H antigen, *Salmonella bonariensis* was used as the source of the "i" component and *Salmonella newport* for phase-2 components. Phases 1 and 2 of *S. newport* and *S. bonariensis*, respectively, were suppressed using "eh" and "enx" antiserums. The antigen was subsequently tested using "eh" and "enx" antiserums to determine the efficacy of the suppression method.

Agglutination Tests.—Tests were set up with both H and O antigens against serums at a finding dilution of 1: 25 and were incubated at 50 C. for four and eighteen hours, respectively. Selected serums were reset in doubling dilutions of 1: 25 through 200. The results of these tests were used as a guide in the selection of reactors for bacteriological examination.

Bacteriological Examination of Reactors to the Agglutination Test.—In no case were the results of the agglutination tests accepted as evidence of infection without confirmation by bacteriological examination of reactors. Liver, spleen, pericardial sac, ovaries, and oviduct were routinely cultured in tetrathionate broth and on the solid mediums indicated below. In addition, crop and intestinal contents were seeded in tetrathionate broth, although selenite mediums were used in some cases for this purpose. Concretions or other lesions were also examined by bacteriological methods. Liver and spleen samples were pooled. Transfers were made to McConkey's, desoxycholate or bismuth sulphite, according to the preference of personnel in the four laboratories conducting the bacteriological examinations. In addition, all tissues and organs were plated directly on McConkey's or other solid selective mediums.

Bacteriological Examination of Poults.—The intestinal tract content was cultured as described for reactors. Livers, lungs, pericardial sacs, and, occasionally, spleens, were cultured on agar slants.

Identification of suspected colonies was completed as described for reactors.

Identification of Cultures.—The behavior of all Salmonella spp. isolated from poults and reactors was determined in dextrose, maltose, lactose, sucrose, mannite, and dulcite. Tests for motility, indol, and hydrogen sulphide production were also completed. Following the preliminary tests, all Salmonella cultures were submitted to the typing center at the University of Kentucky, or to the California State Department of Public Health for further identification by serological methods.

RESULTS

From September, 1949, to April, 1952, the laboratories conducted agglutination tests, using *S. typhimurium* antigens, on 593,341 birds. On 474,135 of this total, considerable field data were collected. These birds were divided into four groups, according to affiliation with organizations. The results of testing on all ranches are summarized in table 8.

TABLE 2—Summary of Agglutination Testing Results, Group 2

	Noninfected ranches				Infected ranches					
Season	No. ranches	No. birds	No. reactors	Reactors (%)	No. ranches	No. birds	No. reactors	Reactors (%)	Total No. ranches	Total No. birds
1950										
1951	24	41,809	309*	0.7	4**	11,684	552	4.7	28	53,493
1951										
1952	24	55,356	82*	0.1	5	17,596	240	1.4	29	72,952

* *Salmonella typhimurium* was not recovered from reactors; ** three flocks sold, no bacteriological examination.

TABLE 3—Summary of Agglutination Testing Results, Group 3

	Noninfected ranches				Infected ranches					
Season	No. ranches	No. birds	No. reactors	Reactors (%)	No. ranches	No. birds	No. reactors	Reactors (%)	Total No. ranches	Total No. birds
1951										
1952	28	40,503	345*	0.85	0	.....	.....	.....	28	40,503

* *Salmonella typhimurium* was not recovered from reactors.

Group 1.—In 1949-1950, group 1 was comprised of 18,091 birds from seven ranches which supplied eggs to one hatchery. It will be noticed in table 1 that infection was found among birds on six of the seven ranches tested during the 1949-1950 season.

It is emphasized, however, that the one negative flock had been hatched in a separate compartment and with extreme care to prevent contact with eggs and poults from the other supply flocks. Inasmuch as a sufficient number of eggs was not available from the single negative flock to supply egg commitments, eggs from infected flocks were also accepted and, as a result, considerable loss in poult mortality was experienced during the 1950 poult season.

In 1950-1951, group 1 was comprised of six ranches with 17,023 birds, table 1. Flocks from five ranches had been tested the previous season while those from the sixth ranch were first tested in 1950-1951. Carriers were found on four of the six ranches; the reactors were removed and the flocks retained for egg production. As was anticipated, losses again resulted during 1951 among poults which were hatched from the infected flocks. Poults from the two negative flocks, however, were brooded without evidence of *S. typhimurium* infection.

During the 1951-1952 season, group 1 was comprised of five ranches with 14,014 birds (table 1). No carriers were found among birds from three ranches, but reactors from the other two ranches yielded *S. typhimurium*. Birds from one of the infected ranches had been shown to be negative in 1950-1951. It was subsequently concluded that infection had been introduced on this ranch in 1951-1952 as a result of importation of breeding stock which was the progeny of birds previously tested and found to be infected. No losses resulting from *S. typhimurium* infection were encountered during the 1952 poult season in progenies of the birds on the three negative ranches. Losses did occur among poults hatched from the flocks shown to harbor carriers.

Group 2.—This group, submitting serums for the first time in 1950-1951, was comprised of 28 ranches and 53,493 birds. Members of this group marketed under an agreement with an egg sales agency which did not attempt to maintain management supervision of the flocks but did require that all prospective breeder birds be negative to the *S. typhimurium* test. Although close field supervision was not afforded the group, attempts were made to familiarize the members with those management features which might influence the *S. typhimurium* status of their flocks. Table 2 indicates that no *S. typhimurium* carriers were found on 24 of the 28 ranches. Birds on one ranch were shown to be carriers of *S. typhimurium*, and the flock was sold. Flocks on three other ranches were found to have titers sufficiently high to indicate the presence of carriers; however, these flocks were sold before an opportunity was provided to select sufficient reactors for bacteriological examination. Therefore, at the end of the testing season there were no

TABLE 4—Summary of Agglutination Testing Results, Group 4

	Noninfected ranches				Infected ranches					
Season	No. ranches	No. birds	No. reactors	Reactors (%)	No. ranches	No. birds	No. reactors	Reactors (%)	Total No. ranches	Total No. birds
1950										
1951	29	56,632	567*	1.0	12	40,346	1,144	2.8	41	96,978
1951										
1952	57	113,129	1,371*	1.2	8	48,252	1,048	2.2	65	161,381

* *Salmonella typhimurium* was not recovered from reactors.

flocks in the group which had not been negative on first test.

It is significant that no isolations of *S. typhimurium* were reported in the progenies of the flocks tested in 1950-1951. The hatcheries which received eggs from group 2 also accepted eggs from other flocks which had not been tested for *S. typhimurium* carriers but apparently hatchery-borne infection did not result.

As with all other groups, members were encouraged to submit poult specimens to the laboratory during the brooding season, particularly in the event of mortality.

During the 1951-1952 season, group 2 was comprised of 29 ranches and 72,952 birds. Of these 29 ranches, 18 had participated in the testing service during the previous season. Birds on 24 of the 29 ranches, table 2, were found to be free from *S. typhimurium* infection.

Reactors from five ranches, with a total of 17,596 birds, were shown to be carriers of *S. typhimurium*. Of these five ranches, all but one had tested the previous year and all had been negative. In four of the flocks toms had been introduced as adults, and it was subsequently established that the flock from which the toms were received had been hatched from eggs of flocks known to harbor *S. typhimurium*. There was no satisfactory explanation to account for the presence of carriers in the fifth flock.

Two of the infected flocks were excluded as suppliers of eggs to group 2, while the remaining three infected ranches continued to act as suppliers after the infected flocks had been sold and replaced with birds negative on the first test. No loss due to *S. typhimurium* infection was reported in progenies of group 2 flocks tested in the 1951-1952 season.

Group 3.—This group, comprised of 28 ranches and 40,503 birds, had for a number of years adhered to a policy which excluded from the egg pool all flocks from which any members of the Salmonella group had been isolated, either as a result of poult autopsy or by examination of reactors to the *S. pullorum* test. Members of the group first participated in the *S. typhimurium* testing service during the 1951-

TABLE 5 — Summary of Agglutination Titer Determinations and Bacteriological Examination of Reactors from Infected Flocks*

Antigen	No. reactors	Titer	No. examined	*Salmonella Typhimurium* recovered (No.)	(%)
"H" only	85	>1:25	14	6	42.9
		1:25	0	0	
"H" and "O"	132	>1:25	35	21	60.0
		1:25	3	1	33.3
"O" only	1111	>1:25	86	32	37.2
		1:25	19	10	52.6

* Complete data from one laboratory.

TABLE 6—Examples of Testing Results on Noninfected Flocks—Serum Titers and Bacteriological Examinations of Reactors

No. birds tested	Titer	ANTIGENS "H" only No. react.	"H" only No. exam.	"H" and "O" No. react.	"H" and "O" No. exam.	"O" only No. react.	"O" only No. exam.	Totals No. react.	Totals No. exam.
1,762	>1:25					3	3	3	3
	1:25					5	2	5	2
712	>1:25	2	2	1	1	8	7	11	10
	1:25					4	0	4	0
1,786	>1:25	7	2			9	3	16	5
	1:25	2	0			9	0	11	0
1,617	>1:25					12	7	12	7
	1:25					9(2)*	0	11	0
3,467	>1:25	3	2			1	1	4	3
	1:25	2	0			1	0	3	0
1,532	>1:25					1	1	1	1
	1:25					1	1	1	1
3,293	>1:25					6	5	6	5
	1:25					8	0	8	0
2,652	>1:25			2	1	21	5	23	6**
	1:25	1	0	1	0	2(6)*	0	10	0
339	>1:25					11	2	11	2†
	1:25					4	0	4	0

* Reactors not titered; ** *Salmonella muenchen*; † *Salmonella san diego*.

1952 season. There were 345 reactors from the flocks of group 3, but *S. typhimurium* was not recovered from the reactors selected for examination (table 3). Eggs from the flocks of this group were sent to 20 hatcheries throughout California. This organization did provide some supervision during the poult brooding season, and their records indicate that little mortality was encountered. In no case was *S. typhimurium* recovered from poults of group 3 during the 1951-1952 brooding season. The apparent value of poult examination and application by the organization of measures for the control of salmonellosis was reflected in the results of the first year's test.

Group 4.—Group 4 included flock owners who were not allied with an organized egg pool. Serums from 258,359 birds were submitted through the seasons 1950-1951 and 1951-1952 (Table 4). The flocks from which the serums were obtained came from 41 ranches during the first year, and from 65 the second. These ranches are classified into subgroups, each of which includes ranches with at least one singular feature which serves to distinguish the subgroup.

Subgroup A.—There were three ranches in this subgroup, and flocks from all three ranches were shown to be infected for two successive seasons. Replacement stock for the second season came from flocks in which there were *S. typhimurium* carriers.

Subgroup B.—There were five ranches in this subgroup. The flocks from all five ranches were shown to have carriers of *S. typhimurium* in 1950-1951, but no infection was found in the replacement stock in 1951-1952. Two of the ranches were restocked with birds from an outside source; the replacements were tested when 5 months old without finding evidence of infection. The remaining three ranches retained the infection breeding stock of 1950-1951, and replacement stock for 1951-1952 was obtained from these flocks. According to the owners, the reactors were removed but the flocks were not retested. The progenies on all three ranches when tested prior to the 1951-1952 laying season showed no evidence of infection. Although

TABLE 7—Examples of Testing Results on Infected Flocks—Serum Titers and Number of Reactors from Which Salmonella Typhimurium Was Recovered

		ANTIGENS							
		"H" only		"H" and "O"		"O" only		Totals	
No. birds tested	Titer	No. react.	No. recov.	No. react.	No. recov.	No. react.	No. recov.	No. react.	No. recov.
1,287	>1:25	3	1/3					3	1/3
	1:25								
1,682	>1:25	2	1/1	1	0/1	20	0/3	23	1/5
	1:25	1				10		11	
1,815	>1:25			1	0/1	15	1/5	16	1/6
	1:25					10		10	
2,379	>1:25					16	4/5	16	4/5
	1:25	3		2	1/2	42(32)*		79	1/2
1,238	>1:25					3	3/3	3	3/3
	1:25	4		1	1/1	8(2)*		15	1/1
1,674	>1:25	5		10	2/2	30	0/2	45	2/4
	1:25	3(28)*		4(15)*		2(150)*	0/1	202	0/1
1,975	>1:25	1		5	1/2	39	0/11	45	1/13
	1:25	1				25(87)*		113	
472	>1:25					14	5/5	14	5/5
	1:25					3(30)*		33	
1,962	>1:25	1		3	2/2	4	2/2	8	4/4
	1:25	2				3		5	
2,817	>1:25			31	5/5			31	5/5
	1:25	1		3		(109)*		113	
2,782	>1:25	3	1/1	13	2/9	13	0/9	29	3/19
	1:25	1		1		6		8	
2,228	>1:25	1		3	0/3	16	1/6	20	1/9
	1:25	2	0/1			16	3/8	18	3/9
53	>1:25			2	1/2	7	1/2	9	2/4
	1:25	1				2		3	
587	>1:25	1	1/1			1	0/1	2	1/2
	1:25								

* Reactors not titered.

these flocks remained free from infection, this result would hardly justify endorsing such a procedure.

Subgroup C.—This group was comprised of six ranches, flocks from which were found to be infected in 1950-1951. The following year flocks on these ranches were not tested.

Subgroup D.—Included in this group are four flocks which were tested for the first time in 1951-1952 and found to be infected.

Subgroup E.—This group was comprised of 59 ranches, with a total of 135,813 birds. Nineteen ranches participated for two consecutive years, submitting serums from 74,629 birds. Forty ranches participated for one year, submitting serums from 61,184 birds. From these two groups the serums of 396 birds reacted with *S. typhimurium* antigen. In no case, however, following examination of representative reactors was *S. typhimurium* recovered.

Antigen-Serum Reactions.—It was shown that those reactors most likely to yield *S. typhimurium* were birds whose serums agglutinated both H and O antigens. This finding is in accordance with observations previously reported by Hinshaw and McNeil.[1] There were, however, ten infected flocks wherein no birds reacted to both the H and O antigens, and of this group, 9 reacted only to O antigen. In only one instance was an infected flock encountered where serum reactions occurred only with H antigen (table 7). These observations would tend to minimize the importance of the H antigen, although in three instances it was necessary to rely on H reactors to detect carriers because all O reactors were examined but failed to yield *S. typhimurium.*

Ten of 19 birds whose serums reacted at less than 1:50 yielded *S. typhimurium* (table 5). The serums of these birds reacted only to the O antigen. In each flock, however, where such reactions were observed, there were other birds in other pens with serums of higher titer.

The serums of 3,092 birds, from 192 flocks in which no infection was found, reacted in the finding dilution (table 8).

From all flocks tested, 874 reactors were submitted for bacteriological examination, and of this group 52.9 per cent were submitted from flocks with no *S. typhimurium* carriers (table 8).

Forty-two per cent of the birds from infected flocks submitted for bacteriological examination yielded *S. typhimurium.*

Although the history of the flock and the low titer of reacting serums often suggested freedom from infection, representative reactors were selected for bacteriological examination irrespective of serum titer. For example, from a total of 15 reactors, 10 were requested for bacteriological examination, but no member of the Salmonella group was recovered (table 6).

Inasmuch as the serum titers in many cases were low, and because of a tendency for serums to drop at least one dilution following overnight storage either at room or refrigerator temperature, it is considered advisable to test serums within twenty-four hours following bleeding.

Reactors from two flocks yielded *S. typhimurium* from the crop only. From one of these flocks, numbering 1,825 birds,

TABLE 8—Summary of Agglutination-Testing Results—All Ranches, 1949-1952 (Three Seasons)

	Noninfected ranches				Infected ranches				Totals		No. reactors examined		
Season	No. ranches	No. birds	No. reactors	Reactors (%)	No. ranches	No. birds	No. reactors	Reactors (%)	Ranches	Birds	Noninfected ranches	Infected ranches	Total
1949													
1950	1	2,752	1	0.03	6	15,339	201	1.3	7	18,091	1	37	38
1950													
1951	89	130,327	1,173	0.9	31	73,747	2,120	2.9	120	204,074	278	172	450
1951													
1952	155	251,132	1,918	0.8	27	120,044	2,165	1.8	182	371,176	183	203	386
Totals		384,211	3,092*	0.8		209,130	4,486**	2.1		593,341	462	412	874

* *Salmonella typhimurium* not recovered from reactors; ** *Salmonella typhimurium* recovered from representative reactors.

there were 10 reactors on the first test. Four of the reactors were selected for bacteriological examination, and *S. typhimurium* was recovered from the crops of 3 of the 4 birds. This flock was retested in forty days and there were only 2 reactors. No member of the Salmonella group was recovered from either of these reactors.

The second flock was comprised of 1,832 birds at the time of the first test; there were 17 reactors, 6 of which were selected for bacteriological examination. Four of the 6 birds yielded *S. typhimurium* from the crop only, but no member of the Salmonella group was recovered from the other 2 birds. This flock was retested in twenty-five days and there were 146 reactors. The data on these two flocks serve to emphasize the importance of including crop and crop contents among those tissues to be examined bacteriologically.

Discussion

Apprehension concerning the efficacy of a serological testing service for the control of *S. typhimurium* has largely been centered around the possibility of infection acquired from exposure to the organism following the test.

Although the results reported herein do not offer a conclusive answer concerning post-test infection, it is possible to make some observations on the problem.

The ability to maintain flocks free from infection following one negative test was demonstrated in the flocks of groups 2 and 3. The progenies of these flocks, when tested the following season, showed no evidence of *S. typhimurium* infection. Turkey production had been practiced on most of the ranches in groups 2 and 3 for from three to ten years.

Although egg and hatchery infection may be controlled, *S. typhimurium* will no doubt, on occasion, be encountered from other sources. That such should occur is not surprising in view of the wide host range of the organism. Infection from these sources may, however, be minimized by excluding, within limits of practicability, opportunity for direct and indirect exposure with known and suspected carriers of *S. typhimurium*.

Retests were not encouraged, except in a few cases where it appeared that retesting of small pens of birds might be of value. Dissemination of the organism by intestinal spreaders, limited duration of the testing season, and testing costs usually tended to discourage retesting.

Hatchery operators were encouraged to improve fumigating methods by increasing the amounts of potassium permanganate and formaldehyde per cubic foot of hatching space, and applying it within a few hours after the eggs were placed in the incubator. Many hatcheries, however, provided two or three additional fumigations in the incubator and hatcher.

Although flock owners of group 3 submitted eggs to 20 hatcheries, there was no evidence of hatchery-borne infection in poults of this group. It may be proposed, however, that the lack of infection did not necessarily imply success in preventing dissemination in hatcheries but rather that infection had probably not been introduced, since no eggs from flocks known to harbor Salmonella carriers had been supplied to the hatcheries. Nevertheless, the fact that evidence of hatchery infection was not encountered does not seem to be without significance.

Many members of the Salmonella group, other than *S. typhimurium*, were recovered from reactors, although some of these organisms had no antigen components in common with *S. typhimurium*.

Summary

The serums of 593,341 turkeys were tested with *Salmonella typhimurium* antigens by the agglutination test during three seasons, 1949-1950 through 1951-1952. There were 384,211 birds in flocks in which no *S. typhimurium* was found. The serums of 7,578 birds reacted in the finding dilution. Of this number, 4,486 were from flocks subsequently proved to have *S. typhimurium* carriers. Forty-two per cent of the reactors submitted from these infected flocks yielded *S. typhimurium*. The other 3,092 birds, whose serums agglutinated one or both antigens, were from flocks eventually considered as noninfected. The tissues of all specimens examined from this group failed to yield *S. typhimurium*.

The O antigen proved the most useful in the detection of carriers. It was found that those reactors most likely to yield *S. typhimurium* were birds whose serums agglutinated both H and O antigens. Most flocks with carriers would have been detected by using only the O antigen; however, the H

reactions were particularly helpful as an aid in selecting reactors most likely to yield *S. typhimurium*.

The significance of low titer reactions was demonstrated in a number of instances wherein birds whose serums failed to agglutinate antigens at 1:50 yielded *S. typhimurium*.

The success of a voluntary *S. typhimurium* detection service is dependent upon compliance with a number of essential requirements, namely: (1) that adequate facilities for bacteriological examination, serological typing, and agglutination work be available; (2) that the management of hatching egg sales groups, the grower and the hatcheryman have some understanding of the problem and a willingness to cooperate in applying fundamental disease control measures.

A testing service is most effective where circumstances permit complete replacement of all flocks shown to harbor carriers of *S. typhimurium*.

Members of two organizations applied those disease control measures that are necessary for the successful operation of a testing service. From these two organizations, flocks of 57 ranches were tested; no losses of poults from *S. typhimurium* infection were encountered following the initiation of the testing service.

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Unusual Pathological Changes in a Case of Fowl Listeriosis

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A REVIEW of the literature on listeriosis (listerellosis) reveals that only 5 cases [1-5] have been previously reported in domestic fowl in this country. Even though this sparsity of reports singularly merits the recording of another case of this infection, the case herein described presents an unusual pathological finding especially for fowl, namely, advanced cirrhosis of the liver, and has an interesting anamnesis.

FLOCK HISTORY

On February 17, 1953, a New Hampshire hen was brought to the laboratories of the Pathological Division for determination of the cause of death. The owner recounted the following story.

The previous October he purchased 12 birds as 4-month-old-pullets. About a month after the purchase 1 of the pullets sickened and died in a few days. Several weeks later 2 more died. By late January, 3 more had succumbed, making the bird submitted for diagnosis the seventh of the original 12-bird purchase to die. In every bird, it had been observed that death was preceded by illness of several days' duration. In this particular hen, it had been noticed that a severe illness occurred about a month prior to death. This abated and the hen appeared normal for several weeks. A recrudescence followed resulting in death.

The flock had free run of the farm and intermingled with other livestock which currently consisted of 10 head of Hereford cattle and 2 sheep. Up to the time of this report, these animals had remained normal. The owner proposed to kill the 5 remaining birds.

MATERIALS AND METHODS

Bacteriological Findings.—The liver and heart's blood were cultured by plunging a hot, inoculating loop into the pulp and ventricle of the organs, respectively, each tissue being streaked on a series of serum agar slants. After twenty-four hours of incubation about a dozen minute bluish colonies, so tiny that they might easily have been overlooked, appeared in pure culture on the streaks made from the liver. None were recovered from the heart's blood. The organism was a gram-positive bacillus. It was agglutinable by listeria antiserum.

Broth cultures incubated for twenty-four hours, and then held for four or more hours at room temperature, contained motile bacilli, but motile organisms were not present in cultures as they were taken from the incubator. These cultures were lightly turbid and tapping the bottom of the tubes caused a filamentous swirl to rise from the bottom.

The organism in mediums containing 1 per cent fermentable substance, 10 per cent filtered horse serum, ½ per cent beef extract, 1 per cent peptone, and Andrade's indicator after forty-eight hours' incubation produced acid without gas from dextrin, dextrose, levulose, maltose, mannose, rhamnose, salicin, and trehalose. Generally by the tenth day glycerol, lactose, and saccharose were fermented. During 16 days of observation arabinose, dulcite, galactose, inosite, inulin, mannite, raffinose, sorbitol, and xylose were not fermented. Neither indol nor hydrogen sulfide were produced. Gelatin was not liquefied. Litmus milk was promptly decolorized beginning at the bottom of the tube and extending upward, a ring of color remaining at the top for several days. After the eighth day of incubation the litmus milk became slowly acidified. Nitrates were not reduced to nitrites.

Beta-hemolytic activity was only satisfactorily demonstrated by pouring plates of blood agar inoculated with very dilute cultures of the organism. The zones of hemolysis thus produced were about 2 mm. in diameter, a tiny lens-shaped colony being in the center.

The biochemical activities of this organism were retested several times and compared by the simultaneous inoculation of a series of the same mediums with two cultures of *Listeria monocytogenes* from the Pathological Division's stock culture collection. One had been isolated from a goat brain in 1939 and the other from a cow brain in 1944. The organism isolated from the hen was consistent in the reactions it produced and these were identical to those produced by the two cultures from the stock culture collection. Trials subsequent to the initial isolation continued to yield small numbers of organisms from a specimen of the liver which was preserved by freezing. Triturating small pieces of the liver with mortar and pestle did not appreciably increase the yield of organisms. Unfortunately, renal tissue was not preserved for culturing.

Pathogenicity for Laboratory Animals.—A 6-hour-old broth culture administered intravenously (0.05 ml. each) to 3 mice via the caudal veins resulted in the death of 2 in forty-eight hours and of the third in seventy-two hours. The organism was readily recovered from both the heart's blood and spleen of these mice.

The same culture was also given intravenously (0.5 ml.) to 1 rabbit and subcutaneously (0.25 ml. each) to 2 guinea pigs. Twelve days after inoculation these animals were killed. The rabbit had lost flesh and was obviously ill, but the guinea pigs

From the Pathological Division, Bureau of Animal Industry, U. S. D. A., Washington, D. C.

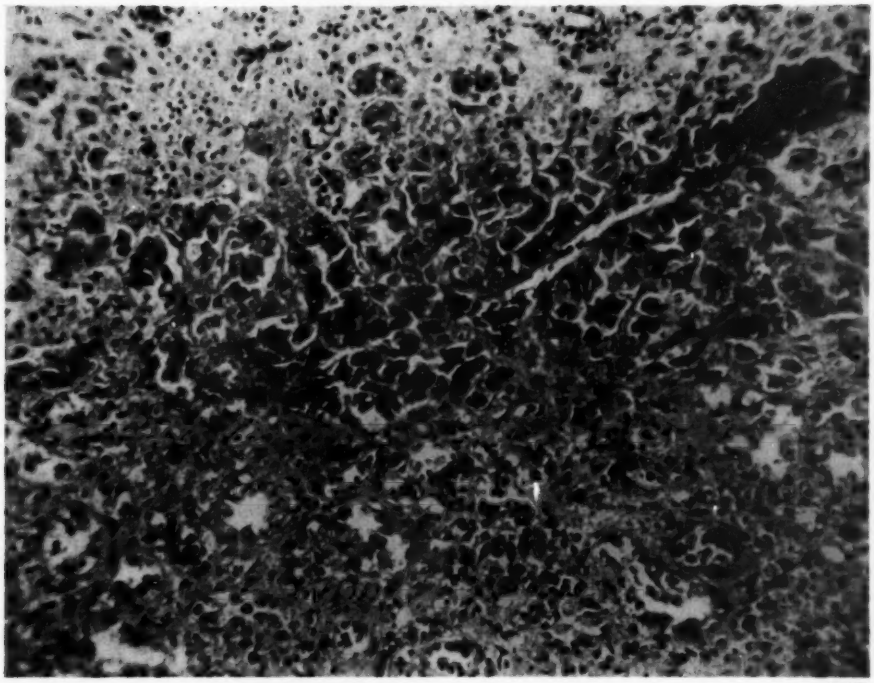

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Fig. 1—Hematoxylin and eosin stain showing dark staining cytoplasm of a cluster of regenerating hepatic cells. Proliferation of bile ducts may be observed in the lower part of photo. Cirrhosis is evident. x 196.

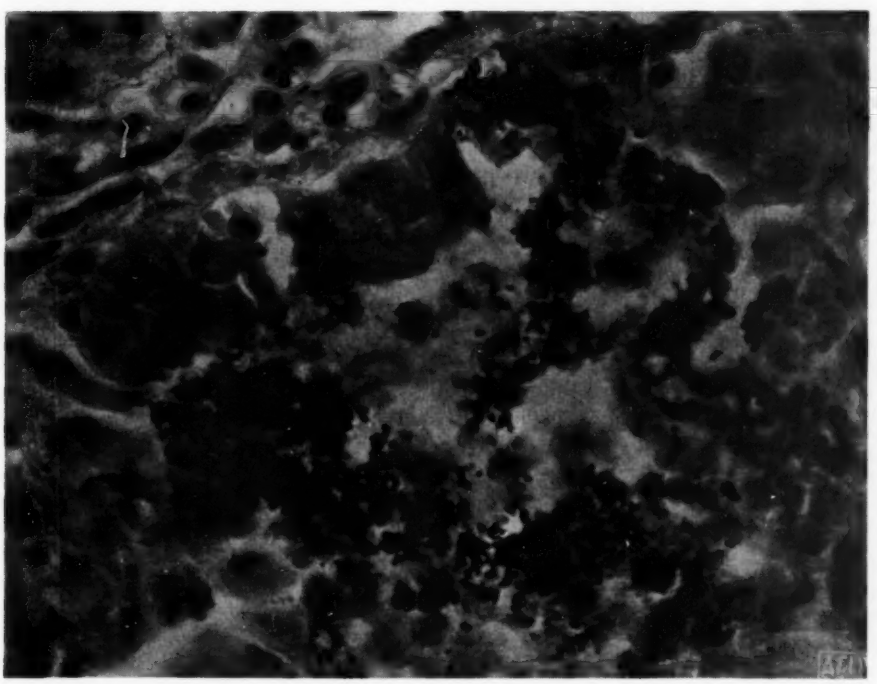

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Fig. 2—Gram's strain superimposed on hematoxylin and eosin. A tubule is shown undergoing degeneration. Colonies of the organism are developing in the disintegrated epithelial cells. x 966.

appeared unaffected. The organism was recovered from both the spleen and liver of the rabbit and the liver of 1 guinea pig.

Serums from the rabbit and the guinea pigs agglutinated a listeria antigen as well as the organism isolated from them.

Gross Pathology.—The specimen was a moderately fleshed hen with no prominent external abnormalities. Removal of the sternum disclosed a markedly enlarged yellowish gray liver which was distinctly fibrous when sheared with the scissors. There were scattered petechiae on the serous surface of the proventriculus and larger, more numerous, and somewhat diffuse, subepicardial hemorrhages. The heart was misshaped and exhibited several large gray areas extending deep into the myocardium. The spleen was enlarged about two times. The kidneys were enlarged, quite firm, and unusually pale in color. Fecal smears were negative for parasite ova.

Portions of the liver, spleen, heart, and kidney were fixed in formalin for histological study.

Histopathology.—Liver.—The most prominent feature of the liver sections was massive fibrosis interspersed with congregations of heterophils. The areas containing hepatic cells were so widely scattered that some searching was required to locate them. Such areas consisted mainly of broken columns of liver cells, many in active regeneration as recognized by the presence of dark staining cytoplasm and occasional binucleated cells. Marked proliferation of the bile ducts was quite conspicuous in many areas of the organ. Even though the organism was isolated from the liver, bacilli were not discernible in Gram's stained sections.

Spleen.—Enlargement of the spleen was primarily due to parenchymatous hyperplasia. The organ was practically devoid of mature lymphocytes. It consisted principally of loosely arranged lymphoblasts. Enlargement may also have been caused by hypostasis which involved one side of the organ.

Heart.—Extensive areas of necrosis occurred throughout the myocardium. The borders of these areas were heavily infiltrated with lymphocytes.

Kidney.—As with the liver, the kidney also contained large areas in which the renal elements had been largely replaced by fibrotic tissue. Degenerative changes, involving the epithelium of many of the tubules, were evident. Casts of degenerated cells occluded and greatly distended numerous tubules. Gram's stain revealed that many of these casts contained colonies of gram-positive bacilli. Several small foci of caseation necrosis bordered by the giant-cell reaction common to fowl tissue were present in the areas of fibrosis.

DISCUSSION

This case presents several interesting aspects. In the first place, it does not appear that the marked pathological alterations of the nature observed in the liver and kidney of this case have been previously associated with listeriosis of fowl. Secondly, the chronicity of the clinical aspects of the case provide evidence that listeriosis may be an infection of long duration in the fowl. The scarcity of organisms in the tissues cultured and the inconspicuous size of the colonies on culture mediums illustrate that the organism might easily be overlooked during busy laboratory diagnostic routines.

In view of the light color of the cirrhotic liver together with its accompanying enlargement, it would be quite possible for the casual observer to mistake such a gross specimen for hepatic lymphomatosis.

It perhaps should be pointed out that *L. monocytogenes* is only motile when grown at room temperature. Lack of knowledge of this fact might easily nullify identification of this organism.

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Fowlpox and the Use of the Whole Embryo Vaccine in Controlling the Disease in Egypt

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FULLY virulent fowlpox virus had been used for many years for vaccination against the disease. Some observations were made on cutaneous application and intramuscular inoculation of the virus. The most common method used at that time was the application of a vaccine prepared from the skin of chickens or squabs infected with fowlpox.

Woodruff and Goodpasture[1] developed the technique of cultivating the virus on the chorioallantoic (c.a.) membrane of the chicken embryo. This method was used by Brandly[2] for the production of a better vaccine from the membrane. Brandly[2] succeeded in cultivating the virus on the c.a. membrane of duck and guinea fowl embryos. Brandly and Dunlap[3] tried to attenuate the fowlpox virus by carrying it for serial passages in the chicken embryos. They reported that, even after 68 passages, the virus showed no apparent reduction in pathogenicity or virulence for the egg or chick. Thorning, Graham, and Levine[4] made a more economical vaccine by cultivating the virus on the c.a. membrane of the chicken embryo and then using the whole contents of the egg for the preparation of the vaccine. Although they found that the highest amount of virus was in the membrane, less in the yolk sac, and least in the embryo, yet the virus titer of seven lots of the whole embryo vaccine was quite high (1:500 to 1:10,000). They also found that the vaccine gave a good proportion of takes (89.7 to 95.5%). Haig[5] reported that, although the whole embryo vaccine gave good takes, the farmers objected to its use because of the presence of small feathers which they thought to be contamination.

In Egypt, fowlpox is considered one of the three important diseases that affect chickens. The other two are fowl plague and Newcastle disease. Fowlpox is more prevalent in the crowded poultry farms, such as those belonging to the breeding department of the Ministry of Agriculture. It has two seasons in this country, one from April to June and the other from October to December, inclusive. In the work that follows, a trial was made to use the whole embryo vaccine in combating the disease in this country.

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MATERIALS AND METHODS

Technique.—The virus used for the preparation of the vaccine was an American fowlpox strain sent to us through the courtesy of Dr. R. A. Alexander of South Africa. This strain was passed for five additional passages on the c.a. membrane of chicken embryos in this laboratory. Three Egyptian fowlpox strains were secured from widely separated outbreaks to be used for challenging experiments. These strains were passed in chicken embryos and tested in susceptible chickens before desiccated stocks were prepared and stored at —20 C. All the eggs and experimental birds used in this work were brought from the breeding

TABLE 1—Titration of Whole Embryo Fowlpox Vaccine in Chickens

Vaccine	Chickens (No.)	Dilution of vaccine	Results on the 10th day
First lot	322 to 324	1:10	Good local reaction; no lesions elsewhere
	325 to 327	1:25	Good local reaction; no lesions elsewhere
	328 to 330	1:100	Good local reaction; no lesions elsewhere
	331 to 333	1:1,000	Good local reaction; no lesions elsewhere
	334 to 336	1:10,000	Good local reaction; no lesions elsewhere
	337 to 338	1:100,000	No reaction until the 3rd week
	339	1:100,000	Slight reaction on the 10th day
Third lot	451 to 453	1:10	Good local reaction; no lesions elsewhere
	454 to 456	1:25	Good local reaction; no lesions elsewhere
	457 to 459	1:100	Good local reaction; no lesions elsewhere
	460 to 462	1:1,000	Good local reaction; no lesions elsewhere
	463 to 464	1:10,000	No reaction until the 3rd week
	465	1:10,000	Slight local reaction; no lesions elsewhere
	466 to 468	1:100,000	No reaction until the 3rd week
Fourth lot	540 to 542	1:10	Good local reaction; no lesions elsewhere
	543 to 545	1:25	Good local reaction; no lesions elsewhere
	546 to 548	1:100	Good local reaction; no lesions elsewhere
	549 to 551	1:1,000	Good local reaction; no lesions elsewhere
	552 to 554	1:10,000	Good local reaction; no lesions elsewhere
	555	1:100,000	Slight local reaction
	556 to 557	1:100,000	No reaction until the 3rd week

poultry farms belonging to the breeding department of the Ministry of Agriculture. There are seven of these farms located in different places in Egypt; each raises from 2,000 to 10,000 chickens

TABLE 2—Cross-Immunity Tests Between the Vaccine Strain and the Egyptian Fowlpox Virus

Chickens (No.)	Dilutions of whole embryo vaccine	Results* of vaccination	Results of challenge with Giza fowlpox virus**
322 to 324	1:10	Good reaction	No reaction
325 to 327	1:25	Good reaction	No reaction
328 to 330	1:100	Good reaction	No reaction
331 to 333	1:1,000	Good reaction	No reaction
334 to 336	1:10,000	Good reaction	No reaction
337 to 338	1:100,000	No reaction	Typical reaction
339	1:100,000	Slight reaction	Slight reaction
664 and 676	Control	.....	No apparent reaction
660, 662, 666	Control	.....	Typical reaction
668, 670, 672	Control	.....	Typical reaction
674, 677	Control	.....	Typical reaction

* Reading of the results of inoculation whether challenge or vaccination was made on the 10th day.

** Challenge with the Egyptian strain was made on the 3rd week after vaccination with the stick method of the wing web.

a year and each is managed by a breeding specialist and a veterinarian.

Preparation of the Vaccine.—Fertile hens' eggs, incubated eleven or twelve days, were inoculated on the c.a. membrane. The inoculation was made by the window method, using hot paraffin for dropping the membrane and scotch tape for covering the window. Each egg received 0.2 ml. of a 1:100 suspension of infected c.a. membrane in saline of the American fowlpox strain. The eggs were reincubated at 36 C. for five days, at the end of which time those living, that showed good lesions on the membrane, were harvested. Dead embryos or those that did not show good pox lesions were discarded. The whole contents of the eggs were collected. To this material was added one quarter of its volume of sterile saline solution. It was then ground in an electric tissue grinder until an even emulsion was made. This material was filtered through one layer of sterile gauze and 1 ml. of the filtrate was put in each ampule. The ampules were put in an Edwards' desiccator until their contents were thoroughly dried under freezing and vacuuming conditions. The tubes were sealed and stored in the refrigerator ready for use.

Titration of the Vaccine in Chickens.—The contents of 1 ampule of vaccine was dissolved in 9 ml. of sterile saline solution to make 1:10 dilution. From this dilution, tenfold dilutions up to 10^{-6} were prepared. Also from the 1:10 dilution, a dilution of 1:25 was made. Each dilution was inoculated into 3 fully susceptible chickens 2 months old, using the stick method of the wing web. The reactions were read on the eighth and tenth day after inoculation but those birds that showed negative results were usually left to the third week after inoculation before they were considered negative. The results are shown in table 1.

As can be seen in table 1, the first and fourth lot of vaccine gave good takes in the chickens inoculated with dilutions as high as 1:10,000, while those which received dilution of 1:100,000 did not show any typical reaction. The third lot gave similar reaction except that of titer 1:10,000 which was not typical. From 1949 on, seven lots of vaccine were titrated and all gave 10^{-4} values except the third lot. The reactions were limited to the seat of inoculation with no secondary lesions found.

Cross-Immunity Tests Between the Vaccine Strain and the Egyptian Fowlpox Virus.—After showing that the vaccine had a high titer, it became of interest to determine whether the vaccine strain of virus would cross immunize against the Egyptian fowlpox strain. The same birds which were used in the titration of the first lot of vaccine were challenged three weeks after vaccination with 1:100 dilution of c.a. membranes infected with the Giza fowlpox virus, using the stick method of the wing web. Ten susceptible controls were inoculated with the same material and in a similar manner. The results are recorded in table 2.

As shown in table 2, the birds which received dilutions of vaccine from 10^{-1} to 10^{-4}, and which showed good takes, did not show any reaction when challenged with the Egyptian strain. Two of the 3 chickens which did not react to the 10^{-5} dilution of vaccine together with 8 of the 10 controls showed typical takes when inoculated with the Egyptian strain. Two of the controls did not show any reaction, which may be attributed to being immune. The third chicken given 10^{-5} dilution vaccine reacted slightly both on vaccination

TABLE 3—Test of Immunity Produced by the Whole Embryo Fowlpox Vaccine Seven Months After Vaccination

Type of birds	Chickens (No.)	Challenge †
Vaccinated birds*	250 to 256	No lesions.
	258 to 261	No lesions.
	263 to 267	No lesions.
	257 and 262	Some papules at the seat of scarification.
	268 to 269	Some papules around the seat of scarification.
Control birds**	223 to 229	Many nodules on the head, eyes, and mouth.
	230 to 234	Few lesions on the head.

* Vaccinated by the stick method with 1:25 dilution of vaccine; ** susceptible birds from Sidse farm; † seven months after vaccination with a 1:100 dilution of chorioallantoic membrane of Egyptian fowlpox virus.

TABLE 4—A Yearly Record of Fowlpox Cases Among the Chickens of the Poultry Farms Belonging to the Ministry of Agriculture

Year	1948		1949		1950		1951		1952	
Farm	Birds (Ave. No.)	Fowlpox cases (No.)	Birds (Ave. No.)	Fowlpox cases (No.)	Birds (Ave. No.)	Fowlpox cases (No.)	Birds (Ave. No.)	Fowlpox cases (No.)	Birds (Ave. No.)	Fowlpox cases (No.)
Saka	10,999	3,689	7,319	200	6,043	0	2,873	0	2,800	0
Sidse	2,008	500	1,652	210	1,436	0	2,096	0	1,055	0
Gimeza	1,624	235	1,316	0	707	3	640	24	472	0
Gizeret El Shieer	—	—	6,582	250	5,627	60	—	—	2,060	4

— Not recorded.

and on challenge. From this experiment, it may be concluded that the American strain of fowlpox which was used in the preparation of the vaccine cross immunizes with the Egyptian strain.

Test of Immunity Produced by the Whole Embryo Vaccine Seven Months After Vaccination.—A flock of 1,500 birds 2 months old on the Enchass farm were vaccinated with 1:25 dilution of the whole embryo vaccine, using the stick method of the wing web. When the birds were examined on the tenth day after vaccination, they showed 98 per cent reactions. Seven months after vaccination, 20 of the birds were brought to the laboratory where they and 12 susceptible controls were given a 1:100 dilution of c.a. membrane infected with Giza strain of Egyptian fowlpox virus. The challenge was made by applying the virus to the scarified combs and wattles of the birds. The results of this experiment are shown in table 3.

From table 3, it can be seen that 4 of the vaccinated chickens showed local nodules at the seat of scarification and the remaining 16 birds proved to be immune to the challenge. Most of the control birds showed pox lesions on the outside parts of the head and a number of them showed diphtheritic lesions at the corners of the mouth and in the throat. From this experiment, it could be concluded that the whole embryo vaccine gave an immunity of about seven months.

Field Application of the Whole Embryo Vaccine.—The vaccine was used in the field by dissolving each ampule in 25 ml. of sterile saline solution, using the stick method of the wing web. This amount of diluent was chosen because it was easy to send anywhere, it gave a better chance for stronger immunity, and it was found to be sufficient to vaccinate at least 500 birds. Birds 2 months old or older were chosen to be the most suitable age for vaccination. The vaccine was first used in the field on small scale experiments. Fifteen hundred 2-month-old native chickens, 2,000 2- to 3-month-old chickens of different breeds, and 1,800 2- to 2½-month-old Fayoumy native chickens and crossbreeds were all vaccinated with the vaccine. These birds were examined ten days after vaccination and 98 per cent of them showed good local reactions. None of these chickens showed any secondary lesions or deviated from the normal state. After these experiments, the vaccine was distributed to the state veterinarians, to poultry farms, and to farmers everywhere in the country. Since 1949, seven lots of vaccine have been prepared. Each lot consisted of 400 ampules and each ampule contained sufficient virus to vaccinate 500 birds, thus making about 1½ million doses of vaccine released to the field.

From the time the vaccine was first used in the field until now, data were collected from the breeding poultry farms belonging to the Ministry of Agriculture. Table 4 represents a yearly record of the total number of chickens and the number of fowlpox cases that appeared among those chickens during 1948 to 1952.

TABLE 5—Annual Record of Fowlpox Cases Among the Chickens of Enchass Farm Before and After Using the Whole Embryo Fowlpox Vaccine

Year	Adult			Young*		
	Birds (Total No.)	Fowlpox cases		Birds (Total No.)	Fowlpox cases	
		recovered	died		recovered	died
1948	2,272	113	27	7,826	4,523	214
1949**	1,701	61	9	7,489	149	212
1950	1,654	19	8	4,332	55	90
1951	1,708	51	4	6,012	66	20
1952	1,609	2	1	7,798	2	2

* Less than 3 months; ** the time at which the vaccine was used on the farm.

As can be seen in table 4, there was a marked decrease in the number of fowlpox cases among the chickens of these farms from the time the vaccine was first used in 1949. In fact, no cases were recorded after the first year of use of the vaccine in the Saka and Sidse farms. On the Gimeza and Gizeret El Shieer farms a few cases were still present after using the vaccine, but they were few as compared to the number of cases recorded before the application of the vaccine.

Since most of the experimental work on the whole embryo vaccine was carried out on the chickens of Enchass farm, a yearly record of fowlpox and the number of birds that recovered and died from the disease in this farm is recorded in table 5. The vaccine was used on the farm at the end of 1949 and the record includes the two years prior, namely, 1948, 1949, and three years after its use was initiated, namely, 1950, 1951, and 1952.

As can be seen in the data of table 5, the number of fowlpox cases among the adult and young chickens on this farm started to show a marked drop from 1950, or one year after using the vaccine. This drop progressed more gradually in the succeeding years until 1952 when only 7 cases of the disease were found on the farm.

DISCUSSION

Fowlpox is one of the three important diseases that affect chickens in Egypt. The disease is widely spread especially in the crowded poultry farms. For many years the disease was controlled in Egypt by using the old tissue vaccine prepared from squabs. From the end of 1949, the whole embryo fowlpox vaccine was used. This latter vaccine gave a high titer, reaching 10^{-4} in chickens and producing a good percentage of takes (98 %). About 1½ million doses of the embryo-propagated vaccine have been used in the field to date and no complaints from mortalities, secondary lesions, or ill after-effects have been recorded. The vaccine was prepared from the whole contents of 11- or 12-day-old chicken embryos infected on the chorioallantoic membrane with an American strain of fowlpox virus. One milliliter of the vaccine was put in each tube and desiccated under vacuum. The vaccine was used in the field by dissolving the contents of each ampule in 25 ml. of sterile saline solution. It was used on chickens by the stick method of the wing web. Two- to three-month-old chickens were the most suitable for vaccination. Data obtained from the breeding poultry farms showed a marked drop in fowlpox cases one year after using the vaccine. In 1952, the disease could be considered under control in these farms as the cases were very few or completely absent.

SUMMARY

1) Fowlpox is one of the three important diseases that affects chickens in Egypt.

2) For many years, the old tissue fowlpox vaccine prepared from squabs was used in controlling the disease in this country.

3) From the end of 1949, the whole embryo fowlpox vaccine was used.

4) The whole embryo vaccine gave a high titer, a good percentage of takes, and there are no ill after-effects.

5) The whole embryo vaccine gave good results in controlling the disease in Egypt. After one year of its use, fowlpox began to decrease and by 1952 cases were few or completely absent.

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Experimental *Vibrio Fetus* Infection in Guinea Pigs
II. Pathological Aspects

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THE GROSS pathological alterations observed in pregnant guinea pigs infected with *Vibrio fetus* were discussed in a previous bacteriological study.[1] The purpose of this paper is to report the histopathological changes observed in the uteri of guinea pigs infected with *V. fetus* and in some of the organs of aborted fetuses.

LITERATURE CITED

Smith[2-5] described placental changes observed in *V. fetus*-infected cows which aborted during the second, or later, pregnancies. An extensive cellular infiltration with subsequent necrosis, edema, and nuclear disintegration was observed in the subepithelial layer of the chorion and in the villi. The endothelium of the capillaries was proliferated. A thrombosed vessel was observed in a villus. In general, sections of the placenta indicated that a considerable amount of normal tissue was still present.

Simon[2] was able to produce acute salpingitis in open heifers. This was the most significant pathological change observed in a heifer slaughtered at sixty-two hours following intrauterine inoculation with *V. fetus*. An extensive generalized inflammatory placental edema, endometritis, and salpingitis were present in pregnant heifers following intrauterine and intravenous exposure to *V. fetus*.

Webster and Thorp[6] demonstrated lesions of the chorioallantoic membranes of embryonating chicken eggs infected with *V. fetus* which resembled the changes observed in the bovine placenta and fetus. There was considerable damage to the fetal endothelial vascular linings with a resultant generalized congestion, hemorrhage, and edema. Hepatic, renal, and splenic enlargement and congestion, as well as evidence of "macrophage mobilization," were frequently observed.

MATERIALS AND METHODS

The following guinea pig tissues were prepared for histological examination: (1) uteri from which *V. fetus* had been isolated; (2) livers and hearts of aborted fetuses from which *V. fetus* had been recovered; (3) identical tissues of control animals and their fetuses.

The tissues were fixed in 10 per cent buffered formaldehyde-saline solution, pH 6.3 to 6.5, transferred through a series of butyl alcohol solutions, embedded in paraffin, and cut at an approximate thickness of 5 μ. Harris' hematoxylin and eosin stains were employed.

RESULTS

Sloughing of the epithelial cells was observed in the uteri of all the guinea pigs infected with *V. fetus*. These lesions varied from a mere breaking away of the epithelial cells to an extensive necrosis and destruction of portions of the endometrium (fig. 1). There was an inflammatory reac-

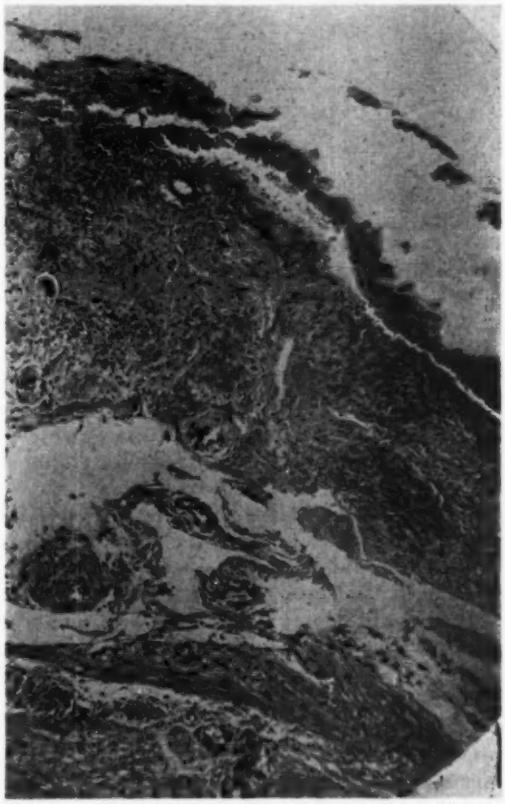

Fig. 1—A section of the uterus of a Vibrio fetus-infected guinea pig. Endometrial destruction and subendometrial edema are present.

Paper N.S. 146 from the Department of Veterinary Science, University of Wisconsin, Madison. Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

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tion characterized by a polymorphonuclear, leukocytic infiltration into the superficial subepithelial region, with many cells throughout the epithelial layer which remained intact. A lymphocytic infiltration of the deeper areas was found in some instances. Small areas of necrosis were observed in the endometrium. These changes were not found in the controls.

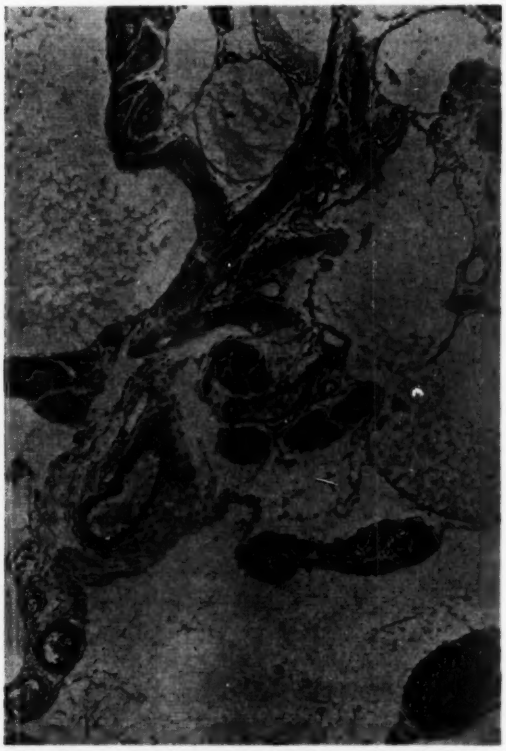

Fig. 2—A section of a Vibrio fetus-infected uterus of guinea pig. Extensive intramuscular edema and enlarged blood vessels are prominent.

Edema was observed in the uterine tissues of all infected animals. It was manifest in the endometrium or, in more extensive areas, in the myometrium (fig. 2). It was present also in the tissues of control animals but this condition was neither as extensive nor as consistent as in the infected group.

Cystic glands were seen in the uteri of some of the infected animals (fig. 3). Enlargement of the uterine glands with occasional periglandular inflammatory cell infiltrations were evident. These alterations were not observed in the controls.

A few thrombosed blood vessels were observed in the uteri of infected animals, but enlarged blood vessels and extensive hemorrhages were commonly present in the uterine tissues of the infected guinea pigs (fig. 1 and 2).

Hemorrhages and enlarged blood vessels were the most noticeable and consistent pathological characteristics of the livers and hearts of aborted fetuses. A slight degree of karyorrhexis was found in some of the liver cells (fig. 4).

DISCUSSION

A marked degree of uterine epithelial destruction was a constant and significant finding in guinea pigs experimentally infected with *V. fetus*. An analogous observation of bovine chorionic epithelium was made by Smith [3] and Simon.[2] It appears reasonable that *V. fetus* travels in the circulatory system from the original portal of entry to the uterus where it causes a circulatory interference of the uteroplacental tissues. Such a process probably results in decreased efficiency of the tissues—an interference with the proper nutritive and excretory functions. The process is a progressive one with denudation of the uterine epithelium and infiltration with polymorphonuclear leukocytes and lymphocytes associated with the circulatory changes.

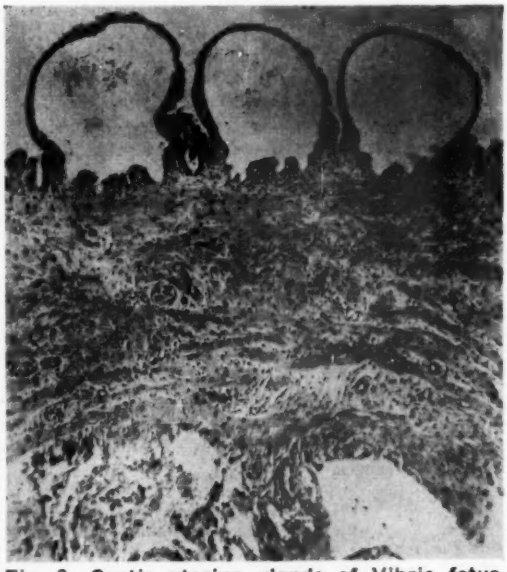

Fig. 3—Cystic uterine glands of Vibrio fetus-infected guinea pig.

Edema, which was a major lesion in the subepithelium and myometrium, was more pronounced in the regions where the greatest erosion of uterine epithelium occurred.

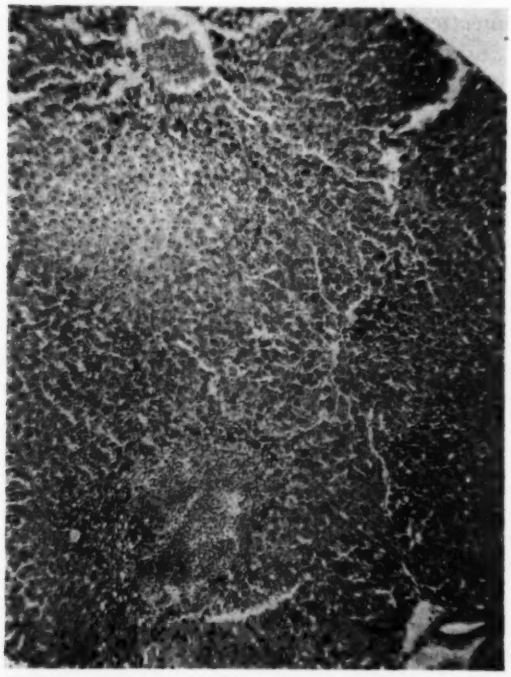

Fig. 4—A section of the liver of Vibrio fetus-infected aborted fetus. Note enlarged blood vessels and hemorrhages into the surrounding tissues.

Since endometrial hemorrhages were not usually associated with inflammatory reactions, it is possible that these were secondary to *V. fetus* infection.

The cystic uterine glands may be associated with a vacuolar degeneration of the mucosa; however, an endometrial gland occlusion may also lead to such a condition.

The results of this study closely resemble the results obtained in the earlier studies of *V. fetus* infection in cattle.[2-5] In some instances, there was a definite similarity of abnormalities following *V. fetus* infection in these two species. This phenomenon suggests that the guinea pig might prove to be a suitable laboratory animal for the experimental study of this disease.

SUMMARY

Characteristic pathological alterations observed in the uteri of guinea pigs infected experimentally with *Vibrio fetus* were epithelial sloughing, inflammatory cell reaction, hemorrhages, enlarged blood vessels, extensive myometrial and subendometrial edema, and cystic uterine glands. The most noteworthy histological changes of the fetal tissues were hemorrhages of the myocardium and liver.

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A Method of Reproducing Teat Topography (Structure) for Evaluation of Teat Erosion

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LITERATURE CITED

Patent teat sphincters have been reported in a high percentage of mastitis cases by Murphy [8] and Stuart and Lancaster,[11] whereas McEwan and Cooper [7] found no connection between the appearance of the orifice and the incidence of infection. Teat lesions and injuries have been associated with a high incidence of mastitis.[1, 2, 4, 6, 11] Espe and Cannon [3] and Kennedy [6] have observed irritation and teat erosion to be associated with machine milking, and other workers[2, 5, 9, 10] have attributed erosions specifically to the high vacuum employed.

The only methods of evaluating teat erosion, known to the authors, are subjective and consist of photographs or the physical inspection method of Udall.[12] In a study of factors which may affect udder health, those practices which contribute to teat erosion should be determined. Finding a method which would allow the simultaneous inspection of the appearance of a teat before and after it had been experimentally subjected to a stimulus which might cause teat erosion would be of value in studies of this type.

This paper describes a method of making teat impressions and the reproduction of the teats from these impressions. This technique may be useful in the evaluation of environmental factors which may contribute to teat erosions.

MATERIALS AND METHODS

The Impression.—The materials necessary for this procedure consist of one 250-ml. beaker, one 100-ml. beaker, 6-in. gauze milk filter discs, a milk sample dipper (approximately 25 cc.), a stirring rod, paper towels, water, and a supply of impression plaster.*

The procedure is as follows: (1) Wipe the teat surface to remove all foreign material and massage the udder until the milk is let down and teats are turgid.

2) Insert a milk filter disc in the 100-ml. beaker as a liner by pressing it against the inside wall of the beaker with the forefingers.

3) Using a dry milk-sample dipper, place 3 volumes (approximately 75 cc.) of dry impression plaster in the 250-ml. beaker, add 2 volumes of water and mix thoroughly with the stirring rod.

4) When the above mixture begins to thicken, pour it quickly into the 100-ml. beaker with the liner in place. Insert the teat into this semiliquid plaster and hold it firmly in place for one to two minutes (fig. 1).

5) When the plaster has become sufficiently hard, it may be observed to pull away slightly from the teat at the surface. The beaker should then be lowered gently until the teat is withdrawn from the impression.

6) Wash the plaster residue from the teat, then compare the impression to the teat; be sure the impression is free from air pockets (fig. 2).

7) Identification can easily be marked on the top surface of the impression before it hardens.

Failure to pour the plaster quickly enough often causes air pockets and difficulty in forcing the teat into the plaster. Holding the beaker near the top and resting the back of the hand against the udder surface reduces movement to a minimum.

The cast.—The materials necessary for making the cast include one 50-ml. beaker, one 100-ml. beaker, a stirring rod, a swab or camel's hair brush, a small hammer, rapid stone,† and a dental vibrator.‡

The procedure is as follows: (1) Take a swab or a camel's hair brush which has been heavily soaped and paint the inside of the impression. This prevents the cast from adhering to the impression.

2) Using 31 parts water and 100 parts rapid stone, add the stone gradually to the water, with continuous stirring.

3) When the stone becomes just thick enough so that it will not pour, a small portion is placed in the impression which is held securely in a metal beaker, or similar container, and the vibrator is turned on (fig. 3).

4) Empty the stone into the impression in small amounts, allowing each portion to be settled by vibration.

5) After the impression has been filled, the identification may be marked on the surface of the cast before it becomes too hard, or it may be written on later with indelible ink.

6) In approximately ten minutes, the impression may be removed from the cast by tapping it with a small hammer until the impression cracks.

From the Departments of Animal Diseases and Animal Industries, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

* Snow White Impression Plaster No. 2 is a product of the Kerr Manufacturing Co., Detroit, Mich.

† Rapid Stone is a product of the Kerr Manufacturing Co., Detroit, Mich.

‡ Toothmaster Vibrator (Model 6-H) is a product of the Toothmaster Co., Racine, Wis.

7) Wash the cast with water to remove any adhering impression plaster (fig. 4).

It is necessary that the stone mixture not be too thin or it will form a cast full of air bubbles which will not be homogenous. Mix just enough stone for a single cast since the casts should be made one at a time.

Trouble was experienced in finding a material which would yield equally satisfactory casts.

Fusible metal 160* made excellent casts but proved to be too expensive. Scrap printer's metal was then used, but it yielded casts with poor detail since it has an extremely low melting point and sets rapidly without showing the fine detail of the impression.

The rapid stone finally adopted for this technique gave results which varied considerably at first because manual shaking was employed. However, uniform results are obtained when the vibrator is employed to shake the stone down into the

* S. S. White Co., Philadelphia, Pa.

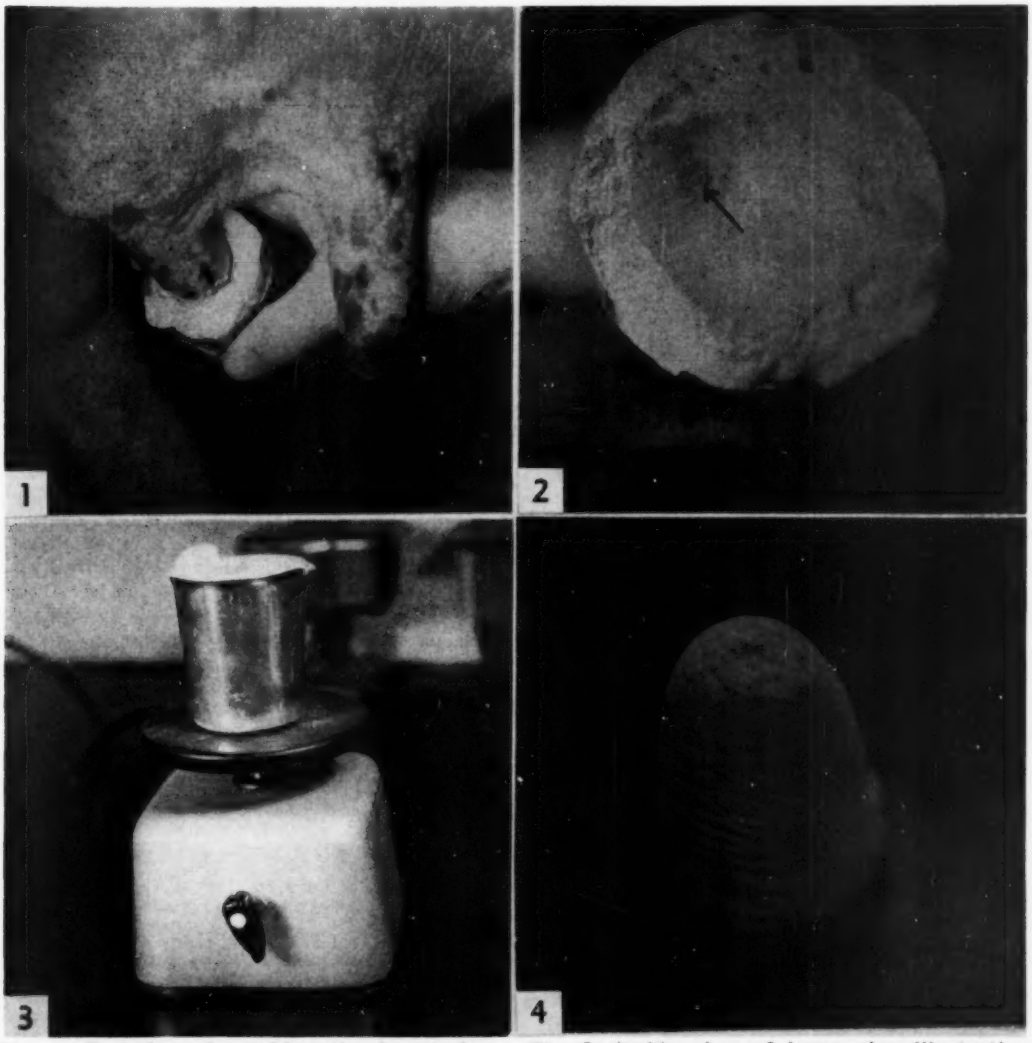

Fig. 1—Technique for taking the impression. As the impression plaster hardens it pulls away from the teat.

Fig. 2—Inside view of impression illustrating the detail of teat end. Arrow indicates external opening of teat canal.

Fig. 3—Vibrator with receptacle containing impression held firmly in place.

Fig. 4—Reproduction of teat. Note the fine detail.

impression. The detail of the casts prepared by this method were comparable to those of fusible metal 160 and were produced at a much lower cost per cast.

SUMMARY

To facilitate research on factors suspected of causing teat erosion, a method is described for making casts before and after a teat has been subjected to a stimulus which might be a factor in causing the erosion.

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Macroscopic Brain Dissection in Veterinary Anatomy

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THOUGH it is generally agreed that working with serial sections is the method of choice for research in the anatomy of the central nervous system, it is doubtful that the student in a basic course gets a tridimensional concept of a relatively complicated organ like the brain. Macroscopic preparations used along with sections and pictures in veterinary anatomy textbooks [1, 4, 14] would aid in teaching the morphology of the central nervous system and give the students a general comprehensive knowledge.

HISTORY

According to Klingler,[6] as early as the first half of the last century Arnold [2] had remarkable pictures of dissected brains. Later on, with the development of the microscopic technique, more emphasis was put on serial sections. In the late 1920's, however, the significance of the macroscopic brain dissection was recognized again by Schlesinger,[18] Hultkrantz,[5] Elze,[3] and Tandler [19]; the latter introduced pictures of dissected brains in his textbook of "Systematic Anatomy."

At the same time Papez [15] dissected brains in this country, using a dissector, orange stick, or some other suitable instrument with a smooth edge. His canine specimens are of special interest in veterinary anatomy. Brain dissection with blunt instruments is also mentioned by Ranson and Clark [16]; Rasmussen [17] used macroscopic brain dissection in an anatomy course at Minneapolis.[20] In working on formalin fixed specimens as it is still done in this country, according to my knowledge, there remains the problem of obtaining the proper consistency.

A technical improvement of the macroscopic brain dissection was made at Basel.[6, 8-10, 12, 13] Klingler [6] refers to a communication of Schmeidel in Vienna, who observed that, by freezing the brain, the contrast between gray and white is much improved. For this reason Klingler started to freeze brains and discovered at once that this treatment led to far better results in dissection. He thinks that the increase in volume of the freezing water in the tissue apparently loosens the fibers without rupturing them.

The brains are taken out of the cranial cavity and suspended at the basilar artery in a low percentage of formalin solution* which fixes all the tissue uniformly and not just that on the surface. Arterial injection is not recommended because better results are obtained when there is more blood in the organ. The brain remains in this fixing fluid for two to three months. Then it is washed in tap water for four to six hours and frozen at 18 to 22 F. or less. After freezing for at least four to five days, it is thawed and may be dissected by removing small pieces of tissue by means of a pair of watchmaker's tweezers. For very fine structures, Klingler sometimes uses feathers of the false wing of snipes. By washing the brain from time to time the dissecting area is kept clean and the whole specimen remains moist, while the gray and the white matter become better defined. The dissecting may be done on whole brains or on blocks of varying size,[6] according to the structures to be shown. Klingler [6] states that the chief value of this method is for didactic purposes,** but it may be a valuable tool for the research worker.

Among veterinary anatomists, Professor Seiferle from the University of Zürich was the first, as far as I know, to realize the importance of specimens of this kind in our teaching. In the summer of 1951, he went to see Dr. Klingler and worked on a human brain. In the fall, arrangements were made which allowed the author to work at Basel, where he had the privilege of being initiated into the macroscopic brain dissection by the highly skilled Dr. Klingler.

TECHNIQUE

To date equine, canine, and bovine brains have been dissected. They were taken out of the skulls from one to two hours after the death of the animals and fixed and frozen according to Klingler.[6] The difference between the gray and the white matter and the dissociation by the freezing process was excellent and the specimens could be dissected easily.

With macrosomatic animals, however, we usually experience trouble since, when taking a fresh brain out of the cranial cavity, we always risk

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This paper reports part of the work done by the author under the supervision of Dr. J. D. Grossman during a stay in Columbus, sponsored by the Institute of International Education and The Ohio State University. He is greatly indebted to the officers of these institutions and to the faculty members of the College of Veterinary Medicine, especially to Dr. Grossman, Dr. H. M. Mauger, and Dr. C. D. Diesem for their kind advice and help in any situation, as well as to Dr. J. W. Papez, Columbus State Hospital, Laboratory for Biological Research, for his interest and his valuable suggestions for further work.

* 5 parts formol, 100 parts water.

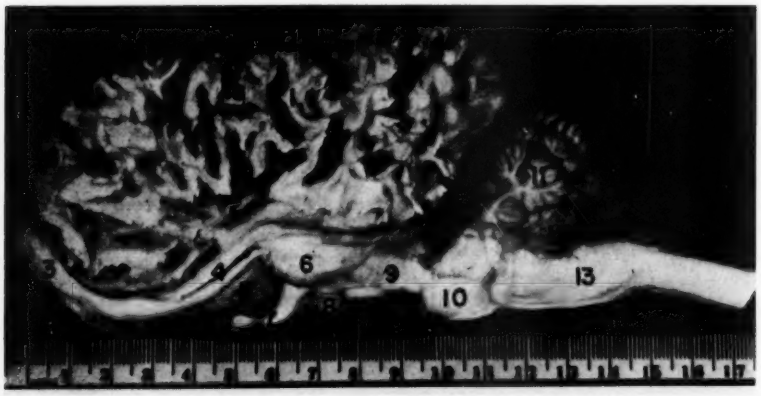
** Compare the pictures of dissected human brains in Ludwig's textbook.[11]

leaving the olfactory bulb, or at least a part of it, in the ethmoid fossa.

As fixed brains are more consistent, formalin was injected into some heads through the carotid arteries, though Klingler[6] does not recommend it. These specimens were intact but, as the blood was washed out, they were pale and there was little contrast between gray and white. Moreover, the dissociation of the fibers was not as good as in the other brains.

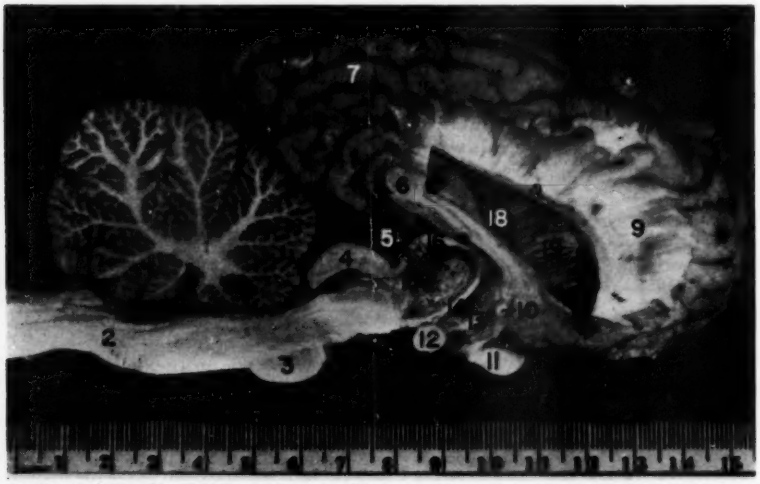
Fixing the brains *in situ* by immersion can not be recommended. Though several openings were made into the cranial cavity by means of a trephine, penetration of formalin was impaired apparently due to the tendency of formalin to swell brain tissue, and the fixation was restricted to small areas around the holes.

Softening the bones with a 10 per cent hydrochloric acid solution, as Dr. Grossman suggested, allows one to obtain complete specimens with relative ease. Since the heads must be left in the HCl-formalin solution for about one to two weeks, they have to be fixed, unfortunately, by injection. If this did not diminish the contrast, this method would be satisfactory, for it is possible to dissect the fibers but, because the gray matter is rather adherent to the underlaying white substance, Klingler's[6] method of fixation is preferred, although it takes much longer to remove the brain from the cranial cavity.



1) Decorticated hemisphere; (2) white matter of insular area; (3) olfactory bulb; (4) lateral olfactory stria; (5) trigonum olfactorium; (6) piriform lobe or area; (7) optic chiasma; (8) mammillary body; (9) cerebral peduncle; (10) pons; (11) cerebellum; (12) choroid plexus of the fourth ventricle; (13) medulla oblongata.

Fig. 1—Lateral view of a horse brain. The left hemisphere has been decorticated in order to obtain a better spatial conception of the sulci and gyri.



1) Cerebellum; (2) medulla oblongata; (3) pons; (4) corpora quadrigemina; (5) pineal body; (6) splenium of corpus callosum; (7) medial surface of hemisphere; (8) cut fibers of corpus callosum; (9) radiation of corpus callosum; (10) anterior commissure; (11) optic chiasma; (12) mammillary body; (13) covered portion of column of fornix; (14) mammillothalamic tract; (15) habenulopeduncular tract; (16) olfactohabenular tract or stria medullaris; (17) hippocampus; (18) choroid plexus of left lateral ventricle (black because of blood congestion and insufficient light); (19) internal capsule.

Fig. 2—Median section of a horse brain. The corpus callosum and the caudate nucleus have been partly removed to show the internal capsule. Some tracts of the diencephalon have been dissected.

The tweezers recommended by Dr. Klingler* were used with a strong, small brush especially for removing the gray matter. Any arrangement which allows washing of the specimens frequently at the dissection site is most convenient.

Fig. 3—Horse brain, posteroventral view. The brain stem along with the cerebellum and a large part of the right hemisphere have been removed.

1) Left cerebral hemisphere; (2) radiation of corpus callosum; (3) cut surface in the diencephalon; (4) piriform lobe or area; (5) trigonum olfactorium; (6) mammillary body; (7) covered portion of the column of fornix; (8) body of fornix; (9) crus of fornix, blending into the fimbria of the hippocampus; (10) stria terminalis; (11) lateral surface of caudate nucleus; (12) anterior commissure; (13) cut end of mammillothalamic tract; (14) cut end of medullary stria or olfactohabenular tract; (15) cingulum; (16) medial longitudinal stria.

APPLICATIONS

Since this paper attempts to acquaint veterinary anatomists with the idea of the macroscopic brain dissection, some applications will be described and a few of the practically unlimited number of specimens possible will be shown in pictures.

Simple decortication, which is made very easy by the dissociation due to the freezing, gives a more plastic view of the sulci and gyri and allows one to see the u-shaped loops of the short association fibers (fig. 1). By going deeper on the lateral side, we can expose the extreme capsule and the claustrum. After removing them, the external capsule becomes visible, and still more medial we will find the putamen and finally the internal capsule seen from the lateral side. Though all of these structures can be studied with relative ease on cross sections, it is much more instructive to observe them in their whole extent at one time.

From the medial side, structures like the stria longitudinalis medialis and the fasciculus cinguli can be dissected. When they are removed the radiation of the corpus callosum is nicely shown. By trimming the horizontal fibers of the corpus callosum, the caudate nucleus can easily be seen, and after removing it the internal capsule is visible from the medial side. There is also no difficulty in dissecting the covered portion of the column of the fornix, the mammillothalamic tract, the stria medullaris or olfactohabenular tract, and the habenulopeduncular tract (fig. 2). The fornix however can be better demonstrated in a specimen made out of an entire brain (fig. 3).

This is especially true for the hippocampus, one of the structures which is very difficult for the student to visualize. If the brain stem with the peduncle, the internal capsule, and the whole neopallium of one side, including the cingulum, is removed, an easy spatial conception of the remaining Ammon's horn is obtained (fig. 4). The anterior commissure with its fibers extending to the olfactory bulb and the piriform lobe or area has been left in place, as well as the mammillary body and the fornix with its continuation into the fimbria of the hippocampus.

These few applications are by no means a complete list. This paper makes no attempt to go into more details and refers the reader to the excellent pictures in the publications of Ludwig,[10, 11] Klingler,[6–8] and Ludwig and Klingler,[12] which are invaluable sources of ideas, when giving the necessary consideration to the basic differences between human and animal brains.

DISCUSSION

The described macroscopic brain dissection is mainly of didactic worth.[6] It is especially valuable for demonstrating structures which, on a two-dimensional section, are not easily understood. Thus, a spatial conception of the central nervous system can be obtained with relative ease.

SUMMARY

1) Attention is drawn to a method of macroscopic brain dissection, which is successfully in use in the Department of (Human) Anatomy of the University of Basel, Switzerland.

2) In comparison to methods used in this country, some differences (dissociation and better differentiation of the fibers and the gray matter by freezing the brain) might be considered as an improvement.

* Dumont tweezers No. 3 used by many watchmakers in this country.

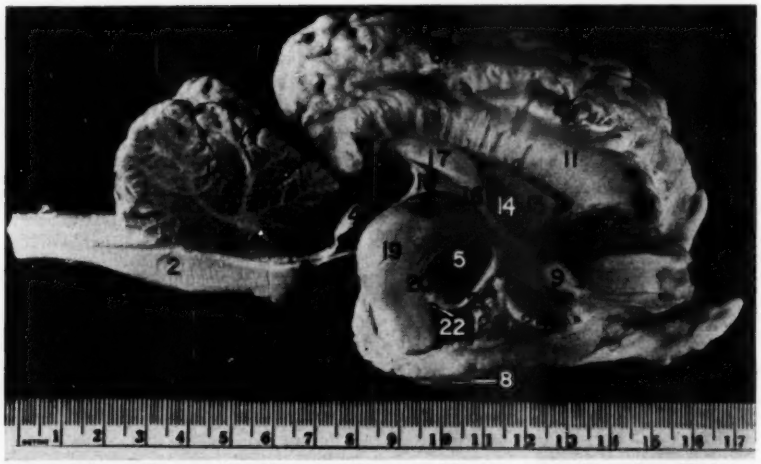


Fig. 4—Horse brain, seen from the right side. The hippocampus, the fornix, and the anterior commissure are shown after removal of the brain stem and the neopallium of the right side, including the cingulum.

1) Cerebellum; (2) medulla oblongata; (3) pons; (4) corpora quadrigemina; (5) intermediate mass; (6) left hemisphere; (7) olfactory bulb; (8) piriform lobe or area; (9) part of the radiation of the corpus callosum of the right hemisphere; (10) cut surface of the precallosal gray and white matter left in place to prevent breaking of the specimen; (11) left radiation of corpus callosum; (12) cut fibers of corpus callosum; (13) caudate nucleus; (14) choroid plexus of the lateral ventricle; (15) septal area; (16) septohippocampal tract; (17) left hippocampus; (18) hippocampal commissure; (19) right hippocampus; (20) fimbria of hippocampus; (21) column of fornix; (22) mammillary body; (23) fibers of the anterior commissure extending to the piriform lobe or area; (24) fibers of the anterior commissure extending to the olfactory bulb.

3) A macroscopic brain dissection of this kind is regarded as being an adequate method of teaching the structures of the brain in veterinary anatomy, since it gives the student an easy tri-dimensional conception of this relatively complicated organ.

4) Some applications are described and shown in pictures.

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The Production and Identification of an Anaplasmosis Complement-Fixing Antibody

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THE PURPOSE of this experiment was to determine the *in vivo* antigenic response to an anaplasmosis complement-fixing antigen. The anaplasmosis antigen was made by the serial passage of *Anaplasmosis marginale* through 3 splenectomized yearling steers, harvested, and prepared by the method described by Price *et al.*[1] This antigen is essentially red blood cell matrices, heavily parasitized by *Anaplasma marginale* bodies and is, therefore, not a homogenous antigenic material. This material has proved to be highly satisfactory in anaplasmosis complement-fixing tests, where the red blood cells matrix content has not proved a serious interference. However, the anaplasmosis antigen has to be considered as consisting of two antigenic portions, one composed of the red blood cell matrices and the other composed of the anaplasma inclusion bodies. The *in vivo* response to such a heterologous antigen would probably consist of antibodies produced against the two portions of the antigen. The problem of determining the *in vivo* antigen's activity of the anaplasma bodies will have to take into consideration the presence of the red blood cell matrix antibodies, which may mask the presence of the anaplasma antibodies.

PROCEDURE

An anaplasmosis antigen and uninfected red blood cell matrices (both prepared in the same manner) were the antigenic materials used in this experiment. These antigens were administered intravenously to separate rabbits in dosages of 5 and 10 mg. of antigen suspended in 1 cc. of physiological saline. The two injections were given three days apart and were followed by four additional intravenous injections of 20 mg. each in 1 cc. of physiological saline, also given three days apart.

The rabbits were bled before and after the injections to obtain serum for titer determinations. The titers were determined by the use of a complement-fixation test, using a protocol supplied by Price.[2] The red blood cell matrices and the anaplasmosis antigen were used in separate tests at a dilution of 1: 12. The titer was determined as the highest dilution of the serum which would give complete fixation of the complement.

After the initial titer determinations, both serums were adsorbed with the red blood cell matrices in order to remove the complement-fixing antibodies for this material. The adsorbtion was accomplished by suspending 50 mg. of lyophilized red blood cell (r.b.c.) matrices in 1.5 cc. of serum, allowing the suspension to stand at room temperature for two hours, then centrifuging at 9,000 r.p.m. for thirty minutes. The supernatant fluid was removed and the same procedure repeated using fresh r.b.c. matrices. The supernatant fluid obtained from the second centrifugation was used for titer determinations.

DISCUSSION

The two unadsorbed serums gave essentially the same titers with both antigens. This result suggests that the titer in each instance was due to the antibody produced in response to the r.b.c. constituent of each antigen. Since adsorption of the two serums with the r.b.c. matrix removed all complement-fixing antibody from the serum of the animal which was injected with r.b.c. but did not remove it all from the

TABLE 1—Results of Complement-Fixation Tests with Rabbit Serums

	Rabbits injected with r.b.c. matrix		Rabbits injected with anaplasma antigen	
Titer	r.b.c. antigen 1 2 3 4*	Anaplas. antigen 1 2 3 4	r.b.c. antigen 1 2 3 4	Anaplas. antigen 1 2 3 4
1: 10	+ + + +	+ + + +	+ + + +	+ + + +
1: 20	+ + + +	+ + + +	+ + + +	+ + + +
1: 40	+ + + +	+ + + +	+ + + +	+ + + +
1: 80	+ + + +	+ + + +	+ + + +	+ + + +
1: 160	+ + + +	+ + + +	+ + + +	+ + + +
1: 320	− − + −	+ − − −	− + − −	− − − −
1: 640	− − − −	− − − −	− − − −	− − − −
Titer after being absorbed twice with r.b.c. matrix				
1: 10	− + − −	− − − −	− − − −	+ + + +
1: 20	− − − −	− − − −	− − − −	+ + + +
1: 40	− − − −	− − − −	− − − −	+ + + +
1: 80	− − − −	− − − −	− − − −	− − − −
1: 160	− − − −	− − − −	− − − −	− − − −
1: 320	− − − −	− − − −	− − − −	− − − −
1: 640	− − − −	− − − −	− − − −	− − − −

* The tests were repeated four times; + complete fixation; − no fixation; r.b.c.—red blood cells.

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The author is deeply indebted to Dr. C. S. McCleskey, professor of bacteriology, Louisiana State University, without whose guidance and advice this experiment could not have been carried out.

serum of the animal injected with the anaplasma antigen, it may be concluded that the remaining antibody is specific for anaplasma. These results indicate that the anaplasma bodies on laked r.b.c. are antigenic in the rabbit.

SUMMARY

1) An anaplasmosis complement-fixing antibody was produced in rabbits by the use of the anaplasmosis complement-fixation test antigen.

2) Concurrently produced complement-fixing antibodies for red blood cell matrices were removed by means of adsorption to permit the identification of the complement-fixing antibodies for the anaplasmosis antigen.

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Influence of Cortisone on Susceptibility of Adult Mice to Foot-and-Mouth-Disease Virus

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FOLLOWING the work of Shwartzman [1] on the enhancement of poliomyelitis infection in mice and hamsters by cortisone, a series of reports concerning the effect of this drug on the infectivity of other viruses has been published.

Kilbourne and Horsfall [2] have demonstrated increased multiplication of influenza viruses, types A and B, and mumps virus in embryonating eggs injected with cortisone. They have reported also that adult mice could be infected with Coxsackie virus, if previously inoculated with cortisone.[3]

Foot-and-mouth-disease virus has been recently shown to infect suckling mice,[4] producing symptoms and lesions which closely resemble those associated with other viruses, including strains of Coxsackie virus. In this laboratory, adult mice have been found resistant to foot-and-mouth-disease virus, although offspring of females exposed to foot-and-mouth-disease virus were immune to infection with the same type of virus as the mother had received, indicating a possible subclinical or inapparent infection in the mother. It was deemed advisable, therefore, to determine if cortisone has any effect on susceptibility of adult mice to foot-and-mouth-disease virus and the results of preliminary experiments are described herein.

MATERIALS AND METHODS

Virus.—A type "O" Vallée strain of foot-and-mouth-disease virus was used in these experiments. This strain originated from a natural outbreak of the disease in cattle in Brazil. Before being used for this experiment, it had been passed once in suckling mice, 16 times in suckling rabbits, and once again in suckling mice.

The infected tissue consisted of the whole carcass less head, skin, and feet of animals destroyed or dead following definite symptoms of disease. Virus suspensions were prepared by grinding infected tissues in a mortar and resuspending in the proper amount of diluent to make a 1:5 suspension. This was centrifuged in a horizontal centrifuge at 3,000 r.p.m. for ten minutes. For passages, the supernatant fluid was mixed with an equal volume of a mixture of penicillin and streptomycin so that the final 1:10 virus suspension contained 1,000 units of each of the antibiotics. This suspension contained 1,000 units of each of the antibiotics. This suspension was incubated in the refrigerator at 4 C. for one-half hour and a portion seeded into fluid thioglycollate medium before inoculation as a sterility test. For titration, serial tenfold dilutions of virus were made in phosphate buffer by first diluting the 1:5 supernatant fluid to make an initial dilution of 10^{-1}. Virus suspensions were inoculated intraperitoneally into both suckling and adult mice in amounts of 0.05 and 0.5 ml., respectively. The inoculated animals were observed for a three-week period and checked daily for clinical signs of infection and mortality.

Cortisone.—Cortisone acetate (cortone acetate Merck) was used. The 25-mg. cortisone tablets were suspended in 2.5 ml. saline (0.85 % NaCl) and 0.5 ml. of this suspension, corresponding to 5 mg. of cortisone was inoculated subcutaneously into each mouse one and one-half to two hours prior to the inoculation of virus. Control animals were inoculated with saline in the same amount and by the same route as those inoculated with cortisone.

Serum Neutralization Test.—Type "O" bovine convalescent and hyperimmune guinea pig serums were used. Normal bovine serum was obtained from the United States. All serums were inactivated at 56 C. for one-half hour and then distributed in 0.2-ml. quantities into Kahn tubes. Serial dilutions of virus were made in phosphate buffer solution. To each tube containing serum, 0.2 ml. of the selected virus dilution was added.

The serum-virus mixture was incubated at 37 C. for one-half hour and 0.05 ml. inoculated intraperitoneally into groups of 4 suckling mice. These animals were observed for fifteen days for symptoms and mortality and the results calculated by the Reed and Muench 50 per cent mortality method.[5]

Complement-Fixation Test.—The complement-fixation test performed with antigens prepared from tissues of adult mice and cattle referred to in these experiments followed the technique already published.[6] Standard types "O," "A," and "C" hyperimmune guinea pig serums were used.

Diluent.—A phosphate buffer stock solution was prepared in accordance with the following formula: $NaH_2PO_4 2H_2O$—2.6 gr.; $Na_2HPO_4 12H_2O$—26.0 gr.; distilled water 1,092.0 ml. When needed,

From the Pan American Foot-and-Mouth-Disease Center, P. O. Box 589, Rio de Janeiro, Brazil; Dr. Eichhorn, director, and Dr. Cunha, chief of laboratories.

TABLE 1—Effect of Foot-and-Mouth-Disease Virus on Adult Mice Treated with Cortisone

Group	Treatment	Mortality according to virus dilution								50 % end point of virus
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
1—adult	Cortisone alone 5 mg.*	.....	.....	.....	.....	.....	.....	.....	.....	.....
2—adult	Cortisone and virus	10/10**	9/10	6/10	5/10	3/10	0/10	.....	.....	$10^{-3.81}$
3—adult	Saline and virus	0/10	0/10	0/10	0/10	0/10	0/10	.....	.....	$<10^{-1}$
4—suckling	Virus	.....	.....	.....	6/6	6/6	6/6	6/6	0/6	$10^{-7.5}$

*—Control group for cortisone. From 30 inoculated mice none died; **—Number of deaths/total injected.

this solution was diluted 1: 20 in distilled water. The final solution maintained a range of pH 7.5-7.7.

Mice.—A Rockefeller Institute strain of Swiss mice was used in all experiments. Those referred to as "suckling mice" were 6 to 8 days old. The "adult mice" were weaned mice, 3 to 4 weeks old, weighing 15 to 17 gr.

Antigens prepared from tissues of this passage were checked by complement-fixation tests and a 70 per cent fixation with type "O" serum was obtained. Two more passages were made in adult mice using cortisone.

In order to determine if direct continuous passage in adult mice had altered the infectivity of the virus in these animals, a virus suspension of

TABLE 2—Mortality Rate of Different Groups of Mice Inoculated with Virus from Tenth Passage

Group	Mortality according to virus dilution								50 % mortality
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Adult mice treated with cortisone	8/8*	8/8	8/8	8/8	8/8	7/8	2/8	.....	$10^{-6.71}$
Adult mice treated with saline	8/8	8/8	8/8	5/8	7/8	2/8	1/8	.....	$10^{-5.44}$
Suckling mice	.....	.....	.....	6/6	6/6	6/6	5/6	0/6	$10^{-7.39}$

* Number of deaths/total injected.

EXPERIMENTS

In the first experiment, three groups of adult mice and one group of suckling mice were used: (1) 30 adult mice inoculated with cortisone only; (2) 60 adult mice inoculated with cortisone and one and one-half hours later with different dilutions of virus suspensions; (3) 60 adult mice inoculated as in group 2 substituting saline for cortisone; (4) five groups of suckling mice inoculated with different dilutions of the virus suspensions.

This data and the results obtained are summarized in table 1.

A tissue suspension from the first passage in adult mice was inoculated into a new group of adult mice previously inoculated with cortisone to make the second passage. This series was continued until the fifth passage. Tissue from the fifth passage was titrated in adult mice previously inoculated with cortisone and in suckling mice. This passage gave titers of $10^{-4.1}$ and $10^{-5.6}$, respectively.

infected tissue from the seventh passage was inoculated into two groups of 16 adult mice, one group of which had received cortisone. Both groups were found to be completely susceptible with a 100 per cent mortality.

Since the virus had apparently been adapted to adult mice, the passages were continued without previous treatment of the mice with cortisone. Tissues from the tenth passage were titrated in three groups of mice: (1) adult mice inoculated with cortisone, (2) adult mice inoculated with saline, and (3) normal suckling mice. The 50 per cent mortality titers were $10^{-6.71}$, $10^{-5.44}$, and $10^{-7.39}$, respectively. The mortality rate of this titration is shown in table 2.

A suspension of tissue from the tenth passage was inoculated into 2 calves which developed typical generalized foot-and-mouth-disease infection. Epithelium from the tongue was harvested from these calves and an antigen prepared for the com-

TABLE 3—Serum Neutralization Test with Mouse-Adapted Virus and Known "O" Serums

	Virus dilut'ons in serum-virus mixture								Results	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	50 % mortality	m.l.d. neutralized
Bovine "O" serum	0/4*	0/4	0/4	0/4	0/4	0/4	.....	.....	$<10^{-1}$	$>10^{6.33}$**
Guinea pig "O" serum	0/4	0/4	0/4	0/4	0/4	0/4	.....	.....	$<10^{-1}$	$>10^{6.33}$
Bovine normal serum	.....	.....	.....	4/4	4/4	4/4	3/4	0/4	$10^{-7.33}$	.....
Saline	.....	.....	.....	4/4	4/4	4/4	1/4	0/4	$10^{-6.66}$	.....

* Number of deaths/total injected; ** Difference between virus titer and serum titer; m.l.d.—minimum lethal dose.

plement-fixation test. One hundred per cent fiixation was obtained with guinea pig type "O" serum and no fixation was observed with types "A" and "C" foot-and-mouth-disease serum or vesicular stomatitis serums, New Jersey and Indiana types.

As a further check on the type of the virus, a serum-neutralization test was carried out in suckling mice using known "O" serums and infected mouse tissue from the tenth passage. The infectivity titer was $10^{-6.66}$ in saline and $10^{-7.33}$ with normal serum, while the two immune serums showed complete protection against all dilutions of the infected tissue suspension. The results are recorded in table 3. The difference observed between normal serum virus mixture and saline virus mixture may be due to protection afforded by the serum during incubation at 37 C.

Clinical Picture in Adult Mice.—In the first passage, adult mice showed first signs of disease in two to seven days as indicated by ruffled hair, abdominal respiration, curved back, and apathy. Excitation, tremors, convulsions, or rigidity were never observed. When stimulated, they moved with difficulty, showing weakness and difficulty in extending the legs. Most of the sick animals died in sternal decubitus. The disease lasted in general twenty-four to forty-eight hours. In a very few cases, clinical signs have been observed for ninety-six hours. After the fifth passage, in animals inoculated with cortisone, the incubation period and the duration of the disease became less variable, all mice dying from the second to the fourth day. This same material, when passed into adult mice without previous inoculation of cortisone, showed an incubation period of three to eight days. However, after a few passages without cortisone, this period was again shortened to two to four days.

Conclusions

1) Normal adult mice may be infected with foot-and-mouth-disease virus and show symptoms and mortality if previously inoculated with 5 mg. of cortisone. This susceptibility is, however, less than that shown by suckling mice.

2) Consecutive passages of the virus in adult mice previously inoculated with cortisone modify the virus in such a way as to become infective for normal adult mice.

3) Serum-neutralization tests in suckling mice and complement-fixation tests with known positive serums show no modification in the type of the virus as a result of mouse passage.

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Common Infections and Disease Conditions Observed in Wild Norway Rats Kept Under Simulated Natural Conditions

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THIS STUDY is based upon the pathological examination of 100 wild Norway rats, the survivors of a colony which had been maintained by Dr. J. B. Calhoun [1] for twenty-seven months in a simulated natural environment for the purpose of studying their behavior. The original 10 colonizers were obtained by Dr. Calhoun from Parsons Island, in the Chesapeake Bay, and were brought to Baltimore, Md., in February, 1947. The animals were kept in a 100-ft. square pen and were protected from stray animals by a rat-proof fence with an electrical fence surmounted on it. The animals were offered food and water but their mode of life was not otherwise altered. The dead and sick animals were not removed from the premises during the behavior study, thus further simulating natural conditions. At the conclusion of the behavior study, the rat colony was destroyed, thus affording an opportunity to study the disease prevalence. The relationship between parasitic and bacterial diseases and the behavior of the different rats in this study will be reported in another paper by Calhoun.

The fact that rats have diseases transmissible to man or act as carriers or intermediate hosts of parasites pathogenic to man lends importance to a study of their naturally occurring infections. Some of the helminthic parasites that occur in both man and rat are Hymenolepis spp., *Trichinella spiralis*, *Capillaria hepatica*, and the cyst of *Echinococcus granulosus*. Some of the infectious diseases that occur in both are: plague, rat bite fever, salmonellosis, leptospirosis, and histoplasmosis.

MATERIALS AND METHODS

During the period May 25, 1949, through June 3, 1949, 100 rats from J. B. Calhoun's colony in Baltimore, Md., were trapped and brought alive to the National Institutes of Health. In this group were 79 females and 21 males; 68 were mature, 24 were young rats under 4 months of age, and 8 were unweaned. The animals were necropsied upon arrival and examined. Fecal pellets obtained from each of the animals were examined for gastrointestinal parasite eggs by means of salt flotation and centrifugalization technique. Fecal samples were also obtained for the isolation of Salmonella spp. This procedure was carried out by inoculating tubes of tetrathionate broth with a sample of feces and, after a 24-hour incubation period, by streaking this broth on brilliant green (1 to 100,000 dilution) agar plates in the usual manner. Three to five Salmonella-like colonies were picked from these plates and inoculated into differential sugars, gelatin, and litmus milk. The gross pathological lesions in each animal were noted and tissues from each animal were saved for histopathological study. No muscles were examined for Trichinella larvae and the blood was not examined for protozoa, but 2 animals were splenectomized and examined for the presence of *Bartonella muris*. Sections of liver and spleen of 64 rats, selected at random, were cultured on Sabouraud's medium for the isolation of *Histoplasma capsulatum* by Dr. C. W. Emmons of the Laboratory of Infectious Diseases, Bethesda, Md. The isolation of *Streptobacillus moniliformis* from the middle ears of 33 rats, selected at random, was done by Dr. Carl L. Larson (now at the Rocky Mountain Laboratory, Hamilton, Mont.).

RESULTS

The results of the salt flotation fecal examinations on the rats are shown in table 1.

All of the animals, with the exception of 8 suckling animals, were infested with one

TABLE 1—Results of the Salt Flotation Fecal Examinations, Showing the Species of Worms Found and the Total Number of Rats that Were infested with Each Species

Species of worms	*Heterakis spumosa*	Hymenolepis spp.	*Strongyloides ratti*	*Syphacia obvelata*	*Trichuris muris*
No. of animals	56	39	16	2	2

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

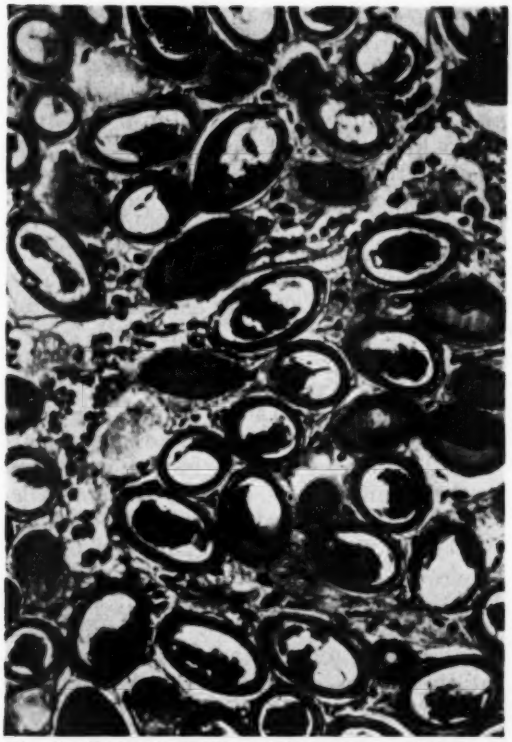

Fig. 1—Eggs of Capillaria hepatica and the cellular infiltration in the liver. x 315.

or more gastrointestinal parasites. *Heterakis spumosa* eggs were encountered most frequently and were present in the feces of 48 mature and 8 young rats. The entire gastrointestinal contents of 6 of the necropsied rats were screened, and the total number of heterakids found in each of the animals was 76, 82, 103, 66, 147, and 144. Also, 10 scolices of Hymenolepis spp. were recovered from 1, but no other gastrointestinal parasites were found in these 6 rats. Hymenolepis spp. eggs were found in 29 mature and in 10 young animals. *Strongyloides ratti* was present in 6 mature and in 10 young rats. *Syphacia obvelata* and *Trichuris muris* were each present in 2 of the mature necropsied rats. Coccidial oöcysts, Eimeria spp., were found in 15 of the 100 rats examined and, with 1 exception, all the infestations were found in young animals. In fecal samples from 3 of the animals, large numbers of coccidial oöcysts were recorded from the salt flotation examinations, indicating severe infections.

The parasite eggs and sections of worms seen in the tissue from the rats are shown in table 2.

Serpentine greyish white markings on the liver due to *Capillaria hepatica* were seen grossly in 35 of the animals. Microscopically, the lesions produced by *C. hepatica* were composed of scattered subcapsular areas of encapsulated masses of brown, oval eggs with bipolar plugs (fig. 1). Occasionally hairlike segments of *C. hepatica* were seen. Early lesions showed necrosis of the hepatic cells, infiltration of eosinophiles and lymphocytes, and macrophages containing considerable quantities of yellowish pigment. Older lesions showed degeneration of the masses of ova, deposition of calcium, and marked proliferation of fibroblasts and fibrosis of the lesions. The nearby bile ducts also showed proliferation and there was moderate increase in fibrous tissue in the portal areas. In one of

Fig. 2—Segments of Trichosomoides crassicauda in the lumen and in the mucosal epithelium of the bladder. x 90.

the rat livers, there was a great increase of dense fibrous connective tissue in the portal areas.

Segments of *Trichosomoides crassicauda* worms were seen both free in the lumen and embedded in the mucosa of the urinary bladder (fig. 2). The characteristic thick-shelled, brown eggs with polar plugs were also seen in the lumen of the bladder. There was no cellular reaction to the parasite or to the eggs.

TABLE 2—Results of the Microscopical Examinations of Sections of Liver, Kidney, Bladder, and Ureter from 100 Rats, Showing the Species of Worms Found and the Total Number of Rats that Were Infested with Each Species

Species of worms	*Capillaria hepatica*	*Trichosomoides crassicauda*	*Cysticercus fasciolaris*
No. of animals	49	6	3

Cysticercus fasciolaris is the larval stage of the tapeworm, *Taenia crassicollis*, of cats. Since 3 cats were able to get into the rats' quarters on an occasion when the electrical fence was not functioning, it is possible that the rats obtained their infection from these animals. However, fecal contamination of the rat feed is another possible source. The larval stage, strobilocercus, within a cyst of the liver is shown in figure 3.

Segments of a small thread-like nematode were found embedded in the mucosa and in the lumen of the ureter at the hilus of the kidney in 2 of the rats (fig. 4). It is possible that these were the larval forms of *Trichosomoides crassicauda* which had migrated from the urinary bladder to the pelvis of the kidney.

Bronchiectasis and abscesses of the lungs were observed in 83.7 per cent of the young and mature rats. This disease was also a common finding in the albino rats and occurred at 48.5 per cent of 103 young and mature albino laboratory rats.[2] The early lesions were confined to the young animals while the more extensive lesions were seen in the older animals. Grossly, the early lesions showed several raised areas on the surface of the lung and small areas of consolidation around the smaller bronchi. Later, single and multiple small abscesses were seen on the basal lobes of the lung. Clinical symptoms—wheezing, sniffling, nasal discharge, encrusted vibrissae—were seen in 30 rats, and these animals showed coalescent abscesses and destruction of the lung tissue of one or more lobes of the lung (fig. 5). On gross section, the different

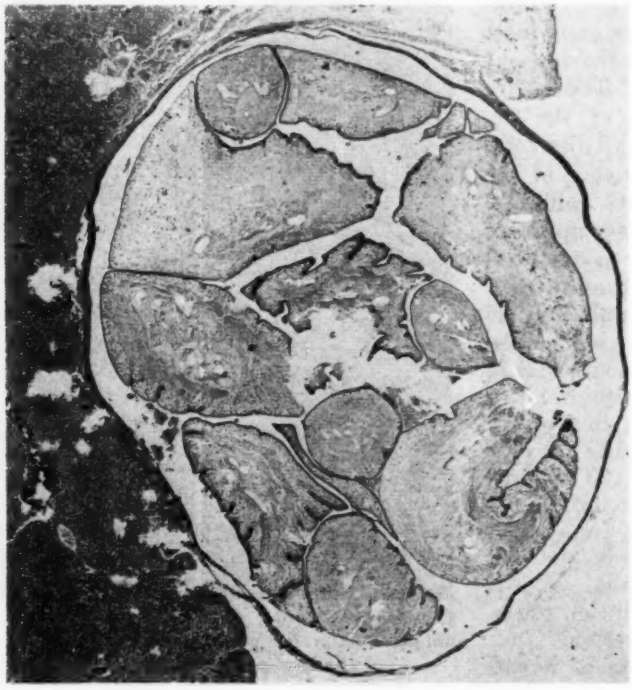

Fig. 3—An encapsulated cyst of Cysticercus fasciolaris in the liver. x 22.

lung lesions showed mucoid or mucopurulent exudate in the bronchi and the nearby areas showed large and small areas containing liquid, mucoid, mucopurulent, inspissated, or caseous material. Some of the areas appeared encapsulated. Microscopically, the early lesions showed a peribronchial infiltration of monocytes and lymphocytes. Advanced lesions were made up of multiple cystlike areas in and around the bronchi with replacement of the bronchial musculature and elastic tissue with fibrous connective tissue. Some lesions showed distention of the bronchi and alveoli with mucoid or mucopurulent exudate and some of the lesions were surrounded by a dense fibrous connective tissue capsule. Hyperplasia of the bronchial epithelium was seen in some of the lungs.

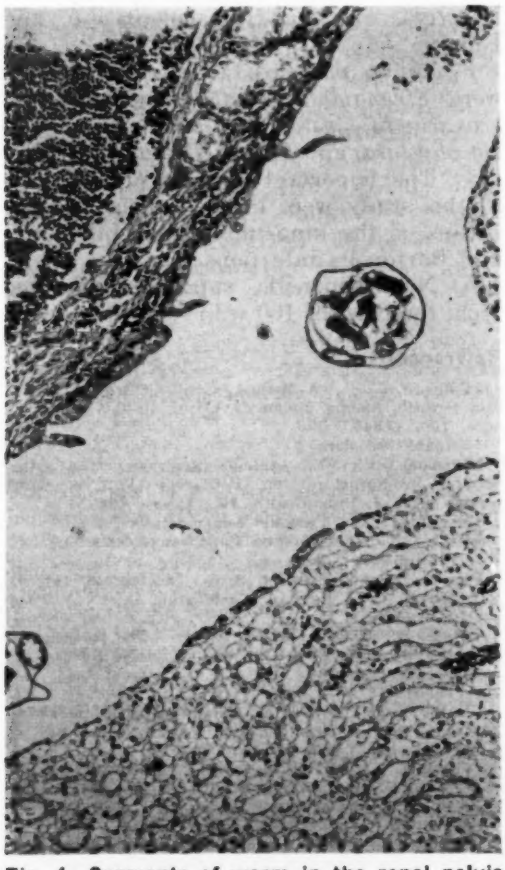

Fig. 4—Segments of worm in the renal pelvis with infiltration of eosinophils and lymphocytes in submucosa and peripelvic areolar tistissue. x 145.

Although *Salmonella enteritidis* and *Salmonella typhimurium* have been occasionally isolated from the feces of albino laboratory rats and wild rats, no Salmonella organisms were recovered from any of the fecal samples of the wild Norway rats in this study.

TABLE 3—The Prevalence of the Cestodes and Nematodes in Wild Rats Surveyed in Three Different Localities

	Locality			
Parasite	D.C.	Tippecanoe Co. Ind.	Baltimore Md.[1]	Baltimore Md.[2]
Cysticercus fasciolaris	54.0	.....	19.2	3.3
Hymenolepis spp.	41.0	7.5	11.2	42.6
Trichosomoides crassicauda	73.0	.....	90.0	6.5
Capillaria hepatica	77.0	.....	85.0	53.3
Strongyloides ratti	.....	.....	20.0	16.4
Nippostrongylus muris	.....	75.0	16.8	.....
Heterakis spumosa	49.0	.....	1.6	60.9
Syphacia obvelata	.....	.....	0.48	2.2
Trichuris muris	.....	2.5	.....	2.2
No. animals examined	100	40	2,500	92

[1] Luttermoser study. [2] Present study.

Following splenectomy of 2 mature rats, the animals showed numerous small coccoid or bacilloid bodies of *Bartonella muris* in a Giemsa-stained blood preparation six days later.

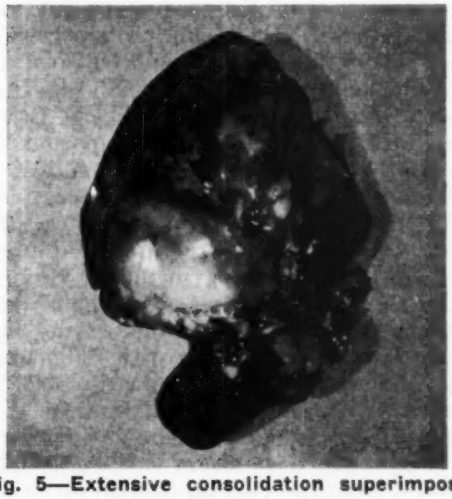

Fig. 5—Extensive consolidation superimposed on bronchiecstasis in right lung.

Emmons [3] reported that no *Histoplasma capsulatum* was isolated from the spleen and liver tissue of 64 rats. Larson [4] isolated *Streptobacillus moniliformis* from the middle ears of 15 of 33 necropsied animals. This organism has been isolated from 41 of 103 albino laboratory rats.[2]

Discussion

Prevalence studies of internal parasites of wild rats, *Rattus norvegicus*, were made in the Washington, D. C., area by Cram [5] in 1928, and Price and Chitwood [6] in 1931; in Tippecanoe County, Indiana, by Cable and Headlee [7] in 1936, and in Baltimore, Md., by Luttermoser [8] in 1936. The percentages of the helminths found in each of the above studies and in the present study are shown in table 3.

The prevalence of parasites in the different studies shows differences which may be due to variations in soil conditions which could effect the development of the larval stages of the parasites or to the availability of the intermediate hosts or to the availability of feed supply. Table 3 shows a similarity of parasites in the sheltered rats obtained from Parsons Island and the wild rats from Baltimore, and Washington, D. C., despite the long geographic isolation and effective isolation from mainland wild rats.

Salmonellosis of rats has been incriminated as a source of outbreaks of food poisoning and food infections in man and has resulted in several studies to determine the prevalence of Salmonella spp. in wild mice and rats. Myer [9] in 1927 reported 28 isolations of *S. enteritidis* and 30 isolations of *Salmonella aertrycke* from 775 rats from San Francisco, Calif., and Welch, Ostralenk, and Bartram [10] in 1941 reported the isolation of Salmonella spp. from five of 420 samples of rat and mouse feces from different parts of the United States. Davis [11] examined 1,386 rats from different locations in Baltimore, including residential areas, poultry houses, a warehouse, and a nearby farm, and found that 4.2 per cent of the rats were infected with Salmonella spp. From these animals he isolated 43 strains of *S. enteritidis*, 12 strains of *S. typhimurium*, one strain of *Salmonella derby*, and one strain of *Salmonella anatum* from the visceral organs, intestinal contents, or both. Davis [12] also made bacteriological examinations for Salmonella spp. and Leptospira spp. from the visceral organs and intestinal contents of 31 male rats obtained from Calhoun's colony of sheltered rats of Baltimore in May, 1949, and found the animals were free of Salmonella spp. and Leptospira spp. Likewise in this study, even though dead animals were not removed from the enclosure and no effort was made to keep birds from contaminating the feed, water, and premises, no Salmonella spp. were isolated from the animals.

Summary and Conclusions

1) Wild rats kept under simulated natural conditions were infested with common parasites that occur in wild rats in the Washington, D. C., area.

2) The most common parasites of wild rats in this study were *Capillaria hepatica*, *Heterakis spumosa*, Hymenolepis spp., and Eimeria spp.

3) Other parasites found in the rats were *Strongyloides ratti*, *Trichosomoides crassicauda*, *Cysticercus fasciolaris*, *Syphacia obvelata*, and *Trichuris muris*.

4) The important diseases of wild rats in this study were bronchiectasis and abscesses of the lung, middle-ear infections, and Bartonella infections.

5) No Salmonella spp. were isolated from the feces of 100 wild rats.

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Response of the Swiss Albino Mouse to Several Virus Strains after Rectal Instillation

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THE VIRUSES employed in this study were the Newcastle disease virus (California strain),[1] Zika and Ntaya viruses,[2] Wyeomyia virus,[3] Bwamba virus,[4] Semliki Forest virus,[5] 17D yellow fever virus,[6] Bunyamwera virus,[7] Ilheus virus,[8] Uganda S virus strain,[9] Anopheles "A" and Anopheles "B" viruses.[3] All of the above viruses, except the Newcastle disease virus strain, were furnished by the American Type Culture Collection in Washington, D. C. (Public Health permit was obtained to work with these viruses.) These viruses were obtained by the above institution from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York, except for the 17D yellow fever strain, which was obtained by the above institution from the Rocky Mountain Public Health Laboratory in Hamilton, Mont., and the Newcastle disease strain, which was obtained originally from the BAI, Department of Agriculture, Washington, D. C.

MATERIALS

Three-week-old Swiss albino mice (Webster strain) were employed in this study. This strain was the one recommended by Webster[10] as being especially susceptible to neurotropic viruses.

Ten, 3-week-old Swiss albino mice were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brain from each strain of the thirty-seventh mouse passage of the Bunyamwera strain, of the 204th mouse passage of the Wyeomyia strain, of the twentieth mouse passage of the Uganda S. strain, of the twenty-sixth mouse passage of the Ilheus strain, of the 165th mouse passage of the Anopheles "A" strain, of the 175th mouse passage of the Anopheles "B" strain, of the eighty-second mouse passage of the Bwamba strain, of the 146th mouse passage of the Zika strain, of the sixteenth mouse passage of the Ntaya strain, of the sixth mouse passage of the Semliki Forest strain, and of the fifth egg embryo passage of the 17D yellow fever strain.

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After an incubation period of between four to eight days, the mice from all groups showed nervous symptoms and mild tremors and paralysis. When symptoms appeared, the sick mice from each group were destroyed and the brains were removed aseptically and kept in separate pools for each strain. Pools of each strain of infected mouse brains were ground with alundum, and were diluted to 20 per cent suspensions with physiological saline. The 11 infected mouse brain pools were then centrifuged for five minutes in an angle centrifuge at 2,000 r.p.m. The supernatant fluids were removed from all 11 pools separately, and the sediments were discarded. Each virus pool was injected intracerebrally into 4 unvaccinated mice and into 4 vaccinated mice. In six to ten days, the unvaccinated mice from each pool showed nervous symptoms; while the immunized mice from each strain appeared normal. This confirmed the identity of each virus used in this experiment. The egg-adapted Newcastle disease virus strain (California strain 11914 egg twenty-fifth passage) was confirmed by neutralization tests conducted in 10-day embryonating chicken eggs. This test was conducted according to the procedure recommended by the Bureau of Animal Industry.[11]

EXPERIMENTAL

Seventy-two, 3-week-old Swiss albino mice were divided into 12 groups of 6 mice each. Each group was administered one of the above stated virus strains by rectal instillation. Each mouse received 0.1 cc. For the rectal instillation, the end of a 22-gauge needle was filed off, rough ends smoothed

TABLE 1—Response of the Swiss Albino Mice to the Following Viruses after the Virus Was Administered Rectally

Virus strain	Min. to max. incubation period (days)	No. mice showing central nervous system involvement	No. mice exposed
Bunyamwera	5 to 6	6	6
Bwamba	0	0	6
Zika	0	0	6
Ntaya	0	0	6
Wyeomyia	0	0	6
Uganda S	9 to 10	6	6
Ilheus	5 to 6	6	6
Anopheles "A"	7 to 8	6	6
Anopheles "B"	0	0	6
17D Yellow Fever	0	0	6
Semliki Forest	4 to 5	6	6
Egg-Adapted N.D.V.	0	0	6

down, and the tip lubricated with vaseline before being inserted. The following mice from virus strain Bunyamwera, Semliki Forest, Ilheus, Anopheles "A" and Uganda S showed symptoms of nervous involvement as shown in table 1, and suspensions made from brains removed aseptically from these five strains were injected intracerebrally into 4 unvaccinated mice and into 4 mice immunized with each respective strain. In four to ten days, the unvaccinated mice from all five strains showed central nervous system symptoms; the immunized mice from all five strains appeared normal. It can be concluded that mice that are inoculated rectally with these five strains succumb to the diseases. The other groups of rectally inoculated mice appeared normal and were discarded after a fourteen-day observation period as shown in table 1.

SUMMARY

Twelve groups of mice were exposed rectally to Bunyamwera, Bwamba, Zika, Ntaya, Wyeomyia, Uganda S., Ilheus, Anopheles "A", Anopheles "B", 17D yellow fever, Semliki Forest, and egg-adapted Newcastle disease virus. Mice infected rectally with the Bunyamwera, Ilheus, Semliki Forest, Anopheles "A", and Uganda S viruses showed nervous symptoms identical to those found in mice infected intracerebrally with these viruses. Mice infected rectally with Bwamba, Zika, Ntaya, Anopheles "B", 17D yellow fever, egg-adapted Newcastle disease, and Wyeomyia viruses appeared normal.

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The Effect of Various Chemical Agents on the Viability of *Mycobacterium Paratuberculosis in Vitro*

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ALTHOUGH *Mycobacterium paratuberculosis* was first observed by Johne and Frothingham [1] in 1895, and was first isolated in pure culture by Twort [2] in 1910, the literature reveals very little data on the resistance of this organism to various chemicals. Further information is lacking on tests carried out on the effect of various chemicals in the presence of organic matter.

Using a culture of *M. paratuberculosis*, Vishnevskii *et al.*[3] found the following chemicals to be bactericidal: *(a)* 2 per cent crystalline carbolic acid solution at two hours' exposure; *(b)* 5 per cent creoline solution in two hours; *(c)* 3 per cent lysol solution in thirty minutes; *(d)* a 5 per cent solution of potassium hydroxide and sodium hydroxide (technical) in twenty-four hours; *(e)* mercuric bichloride in solution 1:500 in ten minutes, or 1:1,000 in one hour; *(f)* 3 per cent formalin solution in twenty minutes. Potassium permanganate had no bactericidal properties in concentrations up to 1 per cent for three hours. They also reported *(a)* that pure cultures of *M. paratuberculosis* when mixed with bovine feces and soil and placed in shade at room temperature (12-13 C.) remained viable for at least eleven months, and *(b)* that when a 3-mg. pure culture of *M. paratuberculosis* was mixed with 1 ml. of physiological saline, sealed in a glass desiccator, and placed near a south window so as to get direct and diffuse sunlight, they were still viable after ten months of exposure.

Lovell *et al.*[4] reported that naturally infected feces may contain viable *M. paratuberculosis* as long as 246 days after exposure to natural atmospheric conditions (26 to 74 F., or −3 to 23 C.), and that intestinal scrapings from cases of Johne's disease may contain viable *M. paratuberculosis* for 163 days after mixing with unsterilized river water. They also found that pure cultures of *M. paratuberculosis* remain viable in either sterilized distilled water, tap water, or pond water for upwards of nine months.

Banerji and Raghavachari,[5] working in India, recovered *M. paratuberculosis* from naturally infected feces after four years of exposure under conditions similar to those in the "field."

Since previous work on chemical treatment has been limited and *M. paratuberculosis* seems to be more resistant than *M. tuberculosis*, it was decided that additional investigations be carried out on the viability of *M. paratuberculosis* following exposure to chemicals under conditions similar to those prevailing in the "field," especially in the presence of organic matter.

MATERIALS AND METHODS

Pure cultures of *M. paratuberculosis* were used in these studies. These cultures were adapted to growing on Dorset and Henley's[6] synthetic medium and on a modification of a bovine lymph gland medium as described by Johnson.[7]

The following chemicals were tested: (1) cresylic disinfectant No. 1, 2, 3, and 4 *; (2) coal tar disinfectant No. 1, phenol coefficient 5; (3) coal tar disinfectant No. 2, phenol coefficient 12; (4) phenol, U.S.P.; (5) sodium orthophenylphenate; (6) formalin; (7) alcohol, ethyl; (8) sodium hydroxide (lye); (9) mercury bichloride, U.S.P. XI; (10) calcium hypochlorite C.P. (70% available chlorine); (11) quaternary ammonium compound No. 1; (12) quaternary ammonium compound No. 2; (13) methylene blue; (14) basic fuchsin; (15) crystal violet; (16) brilliant green.

The relatively high resistance of the species of Mycobacterium to chemical germicides and the difficulties encountered in propagating them on artificial mediums has ruled against the acceptance of any standard *in vitro* test of disinfectants for Mycobacterium species.[8]

Pure cultures of *M. paratuberculosis* used in these experiments were taken from flasks showing maximum growth (fig. 1, A and B).

The chemicals were tested both with and without added organic matter. Two types of tests were carried out with added organic matter. In the first, *M. paratuberculosis* was mixed with a sterile 1 per cent solution of gelatin for one hour before exposure to the chemical. In the second type, the bacteria were mixed with a sterile solution composed of 90 per cent bovine urine and 10 per cent bovine feces and were left standing for one hour before exposure.

The chemicals were diluted to the desired concentration with sterile distilled water and approximately 250 mg. (dry wt.) of the organisms were exposed to 25 cc. of each dilution for periods of fifteen, thirty, or sixty minutes.

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The author expresses his appreciation for the interest and aid given by Dr. Dale A. Porter, director, regional laboratory, and Dr. A. A. Leibold, head professor of bacteriology, School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn.

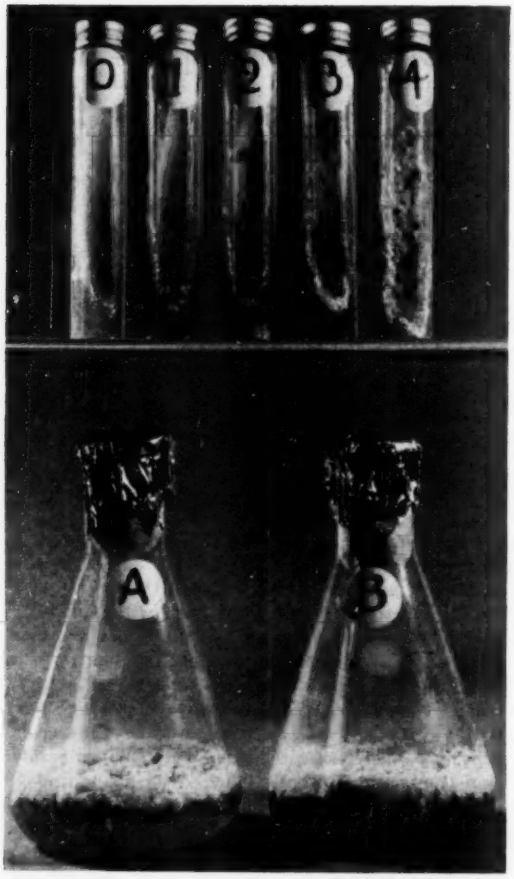
* All cresylic disinfectants listed are permitted by the BAI for use in official disinfection. Each had a minimum of 50 per cent cresylic acid or a minimum of 75 per cent cresylic acid and soap.

At the end of the exposure time the tubes were centrifuged, the disinfectant decanted, and the organisms were thoroughly washed in 25 cc. of sterile physiological saline. Approximately 25 mg. (dry wt.) of the organisms was inoculated on each of six tubes of solid bovine lymph gland medium for each dilution tested and each exposure period. Suitable controls were used for each test.

The inoculated tubes were incubated at 38 C. and observed for evidence of growth for at least three months. If the controls did not show normal growth, the organisms were considered to be of low vitality and the test repeated.

RESULTS

The results of the tests are recorded in table 1. The presence of organic matter did not have any effect upon the germicidal efficiency of the disinfectants tested, except as noted subsequently. The four cresylic disinfectants in a dilution of 1:32 prevented growth of *M. paratuberculosis* when exposed for fifteen minutes.



—Leonard Reid Davis

Fig. 1—Growth of Mycobacterium paratuberculosis on solid and liquid mediums. O = no growth, 1 = poor growth, 2 = fair growth, 3 = good growth, and 4 = excellent growth. A and B shows maximum growth on liquid medium.

It is known [9,10] that the cresols are adversely affected by cooling and dilution and have an odor which is absorbed by food products. However, they are considered one of the disinfectants of choice for *M. tuberculosis* in dilutions of 1:32 and, from results obtained here, they should be equally effective against *M. paratuberculosis.*

The coal-tar disinfectants, phenol coefficient 5 in a 1:64 dilution and phenol coefficient 12 in a 1:100 dilution, prevented growth of *M. paratuberculosis* when exposed for fifteen minutes.

When used in dilutions that prevented growth, it was noticed that cresylic and coal-tar disinfectants seem to lyse some of the bacilli in proportion to the degree of concentration.

Phenol in a 1:40 dilution prevented growth after fifteen minutes of exposure. These results are approximately the same as those described by Vishnevskii *et al.*[3]

Sodium orthophenylphenate in a dilution of 1:100 and 1:200 prevented growth of *M. paratuberculosis* after fifteen minutes of exposure. These results are the same as those that Tilley *et al.*[11] reported effective in preventing growth of *M. tuberculosis.* The advantages of this disinfectant are that it is readily soluble in water, is odorless, is not absorbed by food products, and is nontoxic to higher animals.

Formalin in a 1:25 and 1:33 dilution with an exposure of thirty minutes prevented growth of *M. paratuberculosis,* but the same dilutions did not prevent growth when the time of exposure was fifteen minutes. These results confirm the work of Vishnevskii *et al.*[3] who reported that formalin in a dilution of 1:33 prevented growth of *M. paratuberculosis* when the time of exposure was twenty minutes but did not prevent growth after ten minutes of exposure. Comparative inactivity of formalin was noted by McCulloch and Costigan [12] when testing *Salmonella typhosa,* and by McCulloch and Fuller [13] when testing *Erysipelothrix rhusiopathiae.* In working with *S. typhosa,* McCulloch and Costigan [12] noted that the germicidal power of formalin was influenced slightly by the

TABLE 1—Growth of Mycobacterium Paratuberculosis After Exposure to Various Chemical Agents for Different Periods of Time at 20 C.

Treatment		Dilution	Growth of organisms exposed								
			Without added organic matter			With added organic matter					
						1 % gelatin			bovine 10 % feces 90 % urine		
			(minutes)			(minutes)			(minutes)		
			15	30	60	15	30	60	15	30	60
Cresylic disinfectant	No. 1	1:32	0*	0	0	0	0	0	0	0	0
		1:64	0	0	0	0	0	0	0	0	0
		1:128	1	1	1	1	1	0	0	0	0
	No. 2	1:32	0	0	0	0	0	0	0	0	0
		1:64	3	0	0	4	0	0	1	0	0
		1:128	4	4	4	4	4	4	4	4	4
	No. 3	1:32	0	0	0	0	0	0	0	0	0
		1:64	0	0	0	0	0	0	0	0	0
		1:128	1	1	0	1	1	0	1	0	0
	No. 4	1:32	0	0	0	0	0	0	0	0	0
		1:64	0	0	0	0	0	0	0	0	0
		1:128	4	4	4	4	4	4	4	4	4
Coal-tar disinfectant	No. 1	1:32	0	0	0	0	0	0	0	0	0
		1:64	0	0	0	0	0	0	0	0	0
		1:128	1	1	0	1	1	0	1	0	0
	No. 2	1:100	0	0	0	0	0	0	0	0	0
		1:200	4	4	1	4	4	3	4	1	1
		1:400	4	4	4	4	4	4	4	4	4
Phenol		1:40	0	0	0	0	0	0	0	0	0
		1:80	2	1	1	1	1	1	2	1	1
Sodium orthophenylphenate		1:100	0	0	0	0	0	0	0	0	0
		1:200	0	0	0	0	0	0	0	0	0
		1:400	1	1	1	1	1	1	1	1	1
		1:800	3	2	2	....	....	....	....	....	....
Formalin		1:25	2	0	0	....	....	....	....	....	....
		1:33	4	0	0	....	....	....	....	....	....
		1:50	4	4	1	....	4	....	....	4	....
Alcohol, ethyl		95 %	0	0	0	....	0	....	....	0	....
		70 %	0	0	0	....	....	....	....	....	....
Sodium hydroxide		1:16	1	1	1	1	1	1	1	1	1
		1:20	1	1	1	1	1	1	1	1	1
		1:25	1	1	1	1	1	1	4	1	1
		1:33	2	2	2	4	3	1	4	4	1
		1:50	....	4	2	....	4	....	....	4	....
Mercury bichloride		1:1,000	0	0	0	0	0	0	0	0	0
		1:2,000	3	0	0	1	1	1	4	4	4
		1:4,000	4	4	4	4	4	4	4	4	4
Calcium hypochlorite (70 % Av. Cl.)		1:50	0	0	0	0	0	0	0	0	0
		1:100	1	1	1	0	0	2	3	4	0
		1:200	4	3	2	4	4	4	4	4	4
		1:400	4	4	4	....	....	....	....	....	....
Q.A.C. No. 1 (100 % Av. Quat.)		1:250	1	1	1	2	1	1	1	1	1
		1:500	4	4	2	4	3	3	3	2	1
		1:1,000	4	4	2	4	4	4	4	4	4
		1:2,000	4	4	4	4	4	4	4	4	4
No. 2 (20 % Av. Quat.)		1:125	3	3	3	....	....	....	....	....	....
		1:250	4	4	4	....	....	....	....	....	....
Methylene blue		1:500	0	0	0	0	0	0	0	0	0
		1:1,000	4	3	1	4	3	1	1	1	1
		1:2,000	4	4	1	2	2	2	2	2	1
		1:10,000	4	4	4	4	4	4	4	4	4
Basic fuchsin		1:125	4	4	4	....	....	....	....	....	....
		1:250	4	4	4	....	....	....	....	....	....
		1:500	4	4	4	....	....	....	....	....	....
Crystal violet		1:50	4	4	4	....	....	....	....	....	....
		1:100	4	4	4	....	....	....	....	....	....
Brilliant green		1:33	4	4	4	....	....	....	....	....	....
		1:100	4	4	4	....	....	....	....	....	....

* Growth represented numerically as shown in figure 1; 0 = no growth; 1 = poor growth; 2 = fair growth; 3 = good growth; 4 = excellent growth.

presence of organic matter. Furthermore, they found that the germicidal efficiencies of formalin solutions are influenced by moderate changes in temperature more than are the efficiencies of other commonly used chemical disinfectants. According to the results in table 1, at 20 C. formalin in a dilution of 1:25 can not be recommended as an effective disinfectant against *M. paratuberculosis*. Additional tests carried out at higher temperatures are necessary before a recommendation can be made.

Ethyl alcohol (95 % and 70 %) prevented growth of *M. paratuberculosis* when exposed for fifteen minutes without organic matter; however, alcohol is too expensive to use as a general farm disinfectant.

Sodium hydroxide in a 1:16 dilution did not prevent growth of *M. paratuberculosis* when exposed for sixty minutes. This is approximately the same results reported by Vishnevskii *et al.*[3] when using sodium and potassium hydroxide.

Bichloride of mercury at a 1:1,000 dilution prevented growth of *M. paratuberculosis* when exposed for fifteen minutes, with added organic matter and without added organic matter. A 1:2,000 dilution prevented growth when exposed for thirty minutes without added organic matter, but did not prevent growth after sixty minutes of exposure when organic matter was added. The organic matter apparently reduced the germicidal efficiency. Contrary to these findings, Vishnevskii *et al.*[3] reported growth of *M. paratuberculosis* after exposure to a 1:1,000 dilution of bichloride of mercury for twenty minutes; slight growth after thirty minutes; but no growth after sixty minutes of exposure in the absence of organic matter. They found that a 1:500 dilution prevented growth after ten minutes of exposure.

Although it may be used in the laboratory when working with pure cultures, bichloride of mercury should not be used as a general farm disinfectant because cattle may lick the surfaces on which it has been used and develop mercury poisoning.

Although the hypochlorites are widely used as disinfectants on the farm, they can not be successfully employed against *M. paratuberculosis* because of the high concentration of available chlorine necessary to prevent its growth. It required a 1:50 dilution of calcium hypochlorite or 14,000 parts of available chlorine per 1,000,000 parts of water to prevent growth of *M. paratuberculosis* after fifteen minutes of exposure; whereas, the amount of chlorine used in disinfecting water supplies consists of the application of 1.5 to 10 parts of chlorine per 1,000,000 parts of water,[14] depending upon the offending material in the water. Furthermore, chlorine is a powerful bleaching agent,[14,15] corrodes metals, and its odor is absorbed by food products.

An anomaly occurred with chlorine disinfectants when 10 per cent feces and 90 per cent urine were added for organic matter in that no growth was observed after sixty minutes of exposure at a 1:100 dilution, but growth was observed when no organic matter was added and the organisms exposed for sixty minutes. It is known[12] that chlorine is adversely affected by organic matter. To retain their stability, hypochlorites are prepared at a rather high degree of alkalinity.[14] Carlton and Levine[16] reported better germicidal power as the degree of alkalinity was reduced. Therefore, it is possible that the mixture of feces and urine acted as a buffer in reducing the alkalinity of the chemical to a point where it was germicidal after sixty minutes of exposure, whereas, when no organic matter was added, the chemical remained highly alkaline and growth was observed.

Growth of *M. paratuberculosis* was observed after exposure to a 1:250 dilution of quaternary ammonium compound for sixty minutes. Although the quaternary ammonium compounds are very popular as disinfectants, those used under the conditions of these experiments were not effective. McCulloch *et al.*[17] reported that the quaternary ammonium compounds are inactivated by the presence of manure, blood, milk, tissue debris, and soap.

Of the four dyes tested, methylene blue was the only one that showed any germicidal power in the dilutions tested. Methylene blue in a 1:500 dilution prevented growth of *M. paratuberculosis* when exposed for fifteen minutes, the presence of organic matter having no effect on the germicidal efficiency. Growth was observed at a 1:1,000 dilution after sixty minutes of exposure.

In connection with the experiments on dyes, it was generally observed that in most of the cases where the organisms were

treated with the dyes in low concentrations and transferred to bovine lymph gland medium, the organisms grew at a faster rate than did the controls. It is not known whether the stimulation of growth was due to the presence of an additional favorable nutritive substance, to some physical action of the dyes on the natural waxy coating of the organism, or to some other factor.

SUMMARY

1) The chemical disinfectants that prevented growth of *Mycobacterium paratuberculosis* after fifteen minutes of exposure were: cresylic disinfectants No. 1, 3, and 4 at 1:32 and 1:64 dilution; cresylic disinfectant No. 2 at 1:32 dilution; coal-tar disinfectant No. 1, phenol coefficient 5, at 1:64 dilution; coal-tar disinfectant No. 2, phenol coefficient 12, at 1:100 dilution; phenol at 1:40 dilution; sodium orthophenylphenate at 1:100 and 1:200 dilution; alcohol, 95 per cent, and 70 per cent; mercury bichloride at 1:1,000 dilution; calcium hypochlorite at 1:50 dilution; and methylene blue at 1:500.

2) The chemicals that did not prevent growth of *M. paratuberculosis* after sixty minutes of exposure were: sodium hydroxide at 1:16 dilution; quaternary ammonium compounds at 1:250 dilution of available quaternary; basic fuchsin at 1:125 dilution; crystal violet at 1:50 dilution; and brilliant green at 1:33 dilution.

3) Organic matter reduced the germicidal efficiency of bichloride of mercury. Organic matter did not reduce the germicidal efficiency of the four cresylic acid disinfectants, coal-tar disinfectant No. 1 and 2, phenol, and sodium orthophenylphenate.

4) On the basis of the tests conducted, the disinfectants in the same dilution that are recommended for official use for premises infected with *Mycobacterium tuberculosis* are satisfactory for use for premises infected with *M. paratuberculosis*.

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Morphological Observations by Electron Microscopy of Bwamba Fever Virus after Propagation in the Swiss Mouse

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THE BWAMBA FEVER strain of virus used for this study was furnished through the American Type Culture Collection of Washington, D. C. This virus had originally been obtained from Dr. K. C. Smithburn of the Rockefeller Foundation Laboratories in New York. The Bwamba virus was isolated from the blood of natives in Bwamba, Uganda. This rather mild disease is characterized by fever, headache, conjunctivitis, and skin rash. Recovery occurs after four or five days. The virus was isolated from mice after the intracerebral inoculation of serum from patients. Mice develop an encephalitis after intracerebral inoculation.[1] On receipt at this laboratory, the virus had been passed 82 times in Swiss albino mice by the intracerebral route of inoculation, and it was also passed one additional time before initiating the experiment.

PROCEDURE

Twenty, Swiss albino mice (3-weeks old) were inoculated intracerebrally with 0.03 cc. of a 10 per cent suspension of Bwamba fever virus of the eighty-second intracerebral mouse passage. The virus received here was lyophilized mouse brain and was diluted to a 10 per cent suspension with sterile distilled water. The mice showed nervous symptoms of involuntary motor reactions between the fourth and fifth days. When symptoms appeared, the mice were killed and the brains and spinal cords were removed aseptically. Twenty mice were injected intracerebrally with a 10 per cent suspension of normal mouse brains. No symptoms were noted in these normal controls. These 20 mice were destroyed on the fifth day postinoculation. The brains and cords were removed aseptically as above. The pools of infected mouse brains and cords and of normal mouse brains and cords were ground with alundum and diluted to 10 per cent suspensions with physiological saline. The two suspensions were then subjected to five minutes of centrifugation in an angle centrifuge at 2,000 r.p.m. The supernatant fluids were removed from the specimens and filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation for one hour at 44,770 r.p.m. under refrigeration. The temperature of the refrigerated outer jacket stayed constant at —14 C. during the centrifugation. After the one-hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1.0 cc. of sterile distilled water. A few drops of each suspension were placed on several parlodion film supports which had been prepared forty-eight hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium [2] at arc tangent 2/10 and examined under the RCA electron microscope, type EMU. The balance of the material of each suspension was injected intracerebrally into each of 6, 3-week-old Swiss albino mice.

The mice injected with the concentrated infected material showed nervous symptoms after five days. These mice were destroyed and the brains were removed aseptically, pooled, ground, and diluted to a 10 per cent suspension. This suspension was injected intracerebrally into 6 unvaccinated mice and into 6 mice which previously had been immunized intramuscularly with the Bwamba fever virus strain. After six days, the unvaccinated mice showed symptoms of central nervous system involvement, while the immunized group showed no nervous symptoms. This confirmed the concentrated virus to be Bwamba fever virus. The mice injected intracerebrally with the concentrated suspension of normal mouse brain and cord appeared normal and all of the mice were discarded after a 21-day observation period.

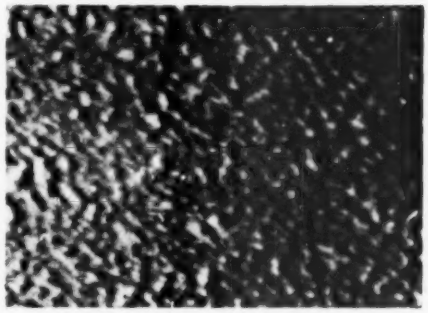

Fig. 1—Concentrated suspension of normal control mouse brain.

From the Virus Department, Livestock Sanitary Service Laboratory, University of Maryland, College Park.

Upon examination with an electron microscope of the concentrated suspension of the brains and cords of the normal mice, no virus-like particles could be seen (fig. 1). spherical, with irregular contour and a diameter of 110 mμ. These bodies could not be demonstrated in concentrated normal mice brains and spinal cords subjected to

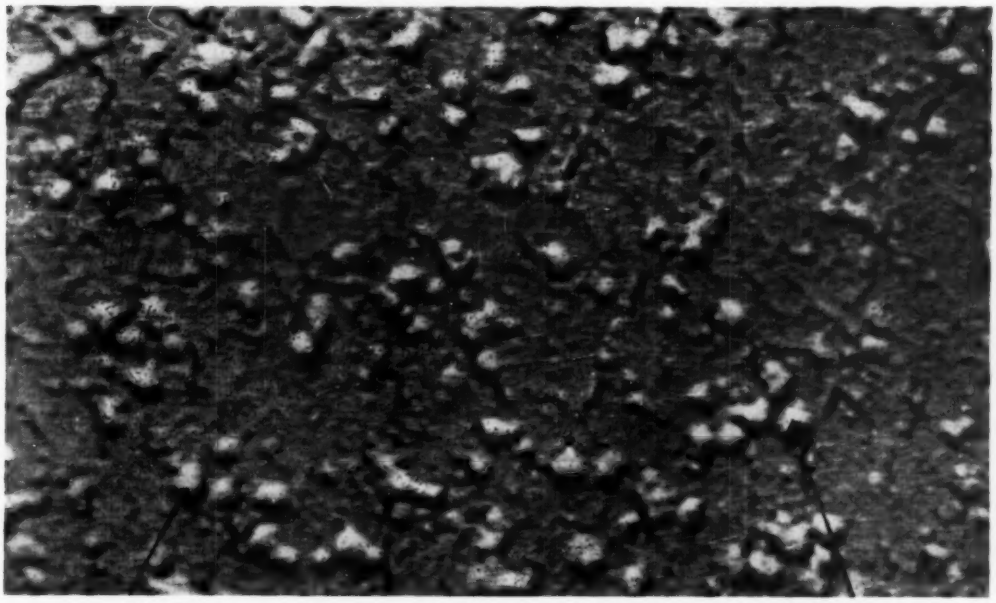

Fig. 2—Bwamba fever virus shadowed with chromium at tangent 2/10. x 54,000.

These controls were screened very carefully. Upon examination of the concentrated suspension from the infected brains and cords, virus-like particles were demonstrated as shown in figure 2. These bodies measure 110 mμ by direct measurement and are spherical with slightly irregular contours.

SUMMARY

Studies by electron microscope of brains and spinal cords of mice infected with Bwamba fever virus show the virus to be the same procedure of concentration and examination. The virus in the concentrated material was infectious for unvaccinated mice but had no effect on mice previously immunized against Bwamba fever virus.

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Electron Micrographs of Erythrocytes from Swiss Albino Mice Infected with the Aujeszky Strain of Pseudorabies Virus

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THE AUJESZKY strain of the pseudorabies virus used for this study was originally isolated in Hungary by Aujeszky [1] and is recognized as identical with the pseudorabies (mad itch) virus causing the disease in the United States. Hurst [2] found rabbits susceptible to this virus when inoculated by peripheral routes, and Shope [3] found guinea pigs and mice susceptible to rabbit-passaged virus. Reagan *et al.*[4] found hamsters and mice also susceptible by peripheral routes to rabbit-passaged virus. The present study was undertaken to see if the virus could be detected on the surface or around the erythrocytes by electron microscopy.

MATERIALS AND METHODS

The virus employed in this study was the Aujeszky strain furnished by the American Type Culture Collection section in Washington, D. C. This virus in infected rabbit brain had originally been obtained from A. Aujeszky in 1931. Before receipt at this laboratory, the virus had been passed several times in rabbits. The exact number of passages is unknown. The glycerinated rabbit brain was ground in a mortar with alundum and diluted to a 10 per cent suspension with physiological saline. This 10 per cent suspension was used as the inoculum throughout the present study.

Three-week-old Swiss Albino mice (Webster strain) were employed. This strain was recommended by Webster [5] as being especially susceptible to neurotropic viruses.

Two guinea pigs were injected intradermally with 0.1 cc. of the inoculum to check the pathogenicity for the specimen. Three days after injection the guinea pigs showed the characteristic symptoms, scratching and biting of the skin at the site of inoculation, excessive salivation, prostration, and death.

EXPERIMENTAL

Thirty-six healthy Swiss albino mice (21 days old) were divided into two equal groups. One group was designated as the control group and the other as the test group. Of the above described inoculum of pseudorabies virus, 0.5 cc. was injected intraperitoneally into each of 18 Swiss albino mice of the test group. Animals of the control group were inoculated intraperitoneally with 0.5 cc. of a 10 per cent brain suspension prepared from normal Swiss albino mice. The animals from each group were further divided into six groups of 3 animals each. Three animals from the control and test group were bled from the heart twenty-four hours postinoculation, drawing approximately 0.1 cc. of blood from each animal. The blood taken from the 3 animals in the test group was pooled and added to 2 ml. of heparinized physiological saline. The blood from the 3 animals of the control group was treated in the same manner. The erythrocyte suspension from each group was then further diluted by adding 2 cc. of physiological saline to each specimen. The cell suspension from each group was then employed for electron microscopic studies. The same procedure was carried out on erythrocyte suspensions taken from mice of the test and the control groups at time intervals of 30, 48, 54, 72, and 92 hours postinoculation. Only animals from the test group at the 92-hour interval exhibited symptoms of central nervous system involvement such as that found in mice infected intracerebrally with the virus strain. The mice from the control group showed no symptoms of nervous system involvement.

TABLE 1 — Response of Swiss Albino Mice Injected with Erythrocyte Suspension from Swiss Albino Mice Infected with Pseudorabies Virus (Aujeszky Strain)

Bleeding interval (hr.)	Mice inoculated with erythrocyte suspensions (No.)	Mice showing symptoms (No.)	Min. to max.* incubation period (days)
24	6	5	3 to 5
30	6	6	3 to 4
48	6	6	3 to 4
54	6	6	3 to 5
72	6	6	3 to 5
92	6	6	3 to 5

* Time elapsing between inoculation and onset of symptoms.

Each specimen was prepared for electron microscopic examination by placing small drops of the erythrocyte-heparinized saline suspension on parlodion film supports which had been prepared forty-eight hours previously. The films were dried and shadowed with chromium [6] at arc tangent 3/14

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park.

Major Reagan is professor of virus diseases; Mr. Delaha, virologist; Miss Stewart, virologist; and Dr. Brueckner, director of the laboratory.

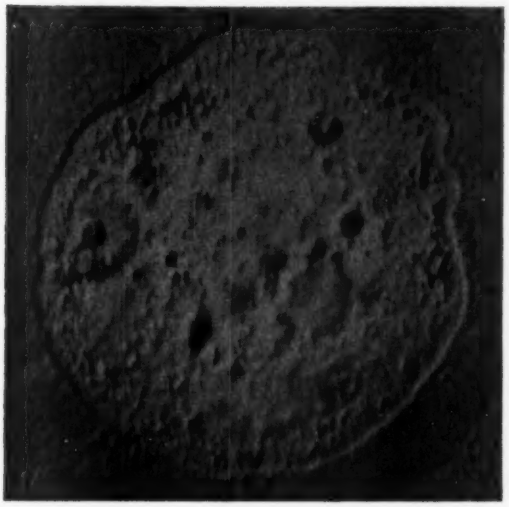

Fig. 1—Electron micrograph of an erythrocyte from mice injected with normal mouse brain from a 48-hour interval of bleeding. Shadowed with chromium at arc tangent of 3/14. x 24,000.

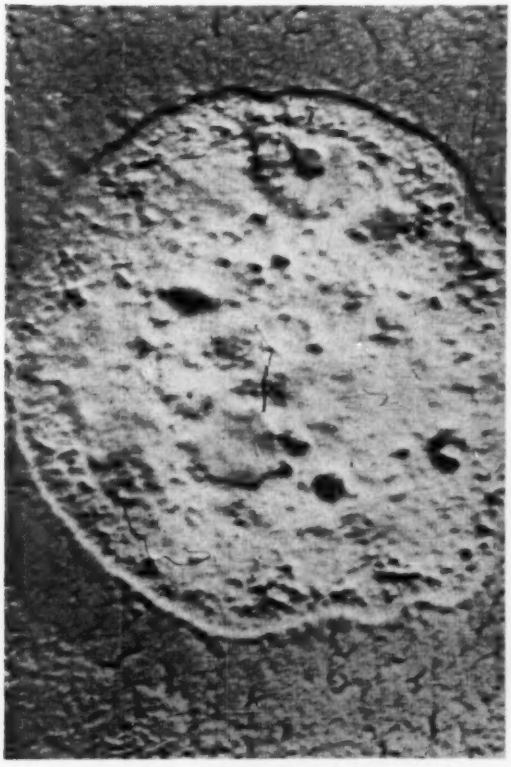

Fig. 2—Electron micrograph of an erythrocyte from mice injected with normal mouse brain from a 48-hour interval of bleeding. Shadowed with chromium at arc tangent of 3/14. x 30,000.

and examined under the R.C.A. electron microscope, type E.M.U.

In order to determine the infectivity of the erythrocytes obtained from animals of the test group, 36 Swiss albino mice were divided into six groups of 6 mice each, and the mice from each group were inoculated intracerebrally with 0.03 cc. of an erythrocyte suspension from a designated time interval. In order to determine the presence of any toxic material in the diluent (heparinized saline), each of 6 normal Swiss albino mice was inoculated intracerebrally with 0.03 cc. of an erythrocyte heparinized saline suspension. The erythrocyte specimen was taken from the mice of the 48-hour control group. After inoculation, the mice were observed twice daily over a 21-day period.

RESULTS

Upon examination by electron microscopy of the erythrocytes taken at desig-

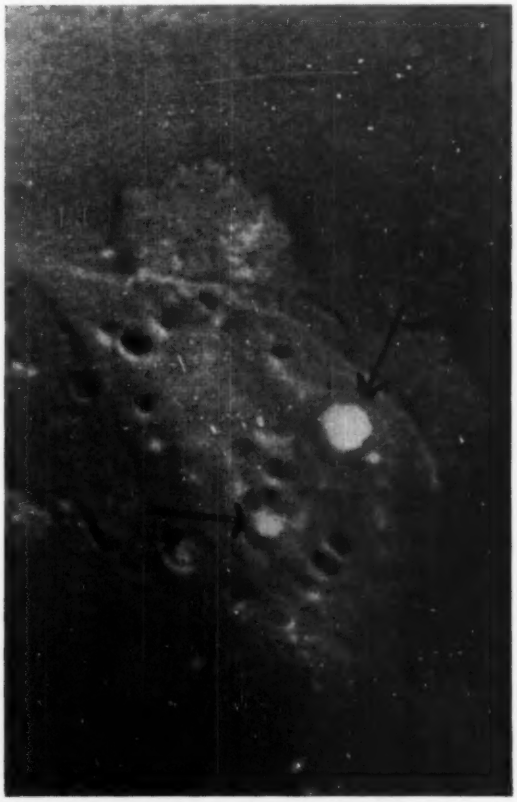

Fig. 3—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval of twenty-four hours. Virus-like particles are indicated by arrows. Shadowed with chromium at arc tangent 3/14. x 30,000.

Fig. 4—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval of thirty hours. Virus-like particles are indicated by arrows. Shadowed with chromium at arc tangent of 3/14. x 30,000.

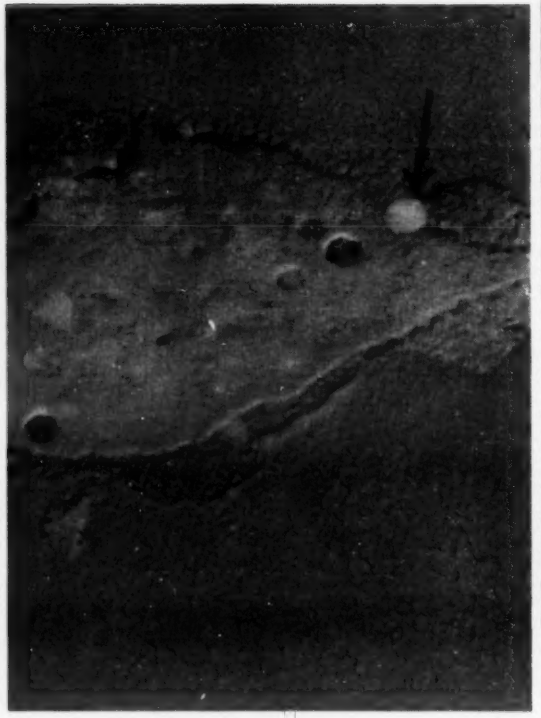

Fig. 5—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval of forty-eight hours. Virus-like particles are indicated by arrow. Shadowed with chromium at arc tangent of 3/14. x 30,000.

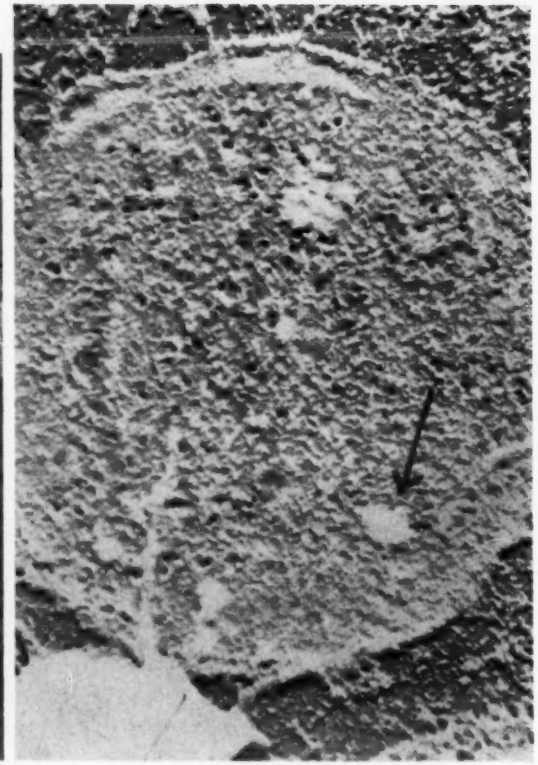

Fig. 6—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from time interval of fifty-four hours. Virus-like particles are indicated by arrow. Shadowed with chromium at arc tangent of 3/14. x 30,000.

nated time intervals from the control group, no virus-like particles could be detected as shown in figures 1 and 2. Micrographs from other control intervals are not included because no virus-like particles were seen. However, upon examination of the erythrocyte suspension taken from the infected Swiss albino mice, a few virus-like particles were seen on the surface of the erythrocytes obtained at the following time intervals: 24, 30, 48, 54, 72, and 96 hours.

These viruslike particles had the same shape and size as those demonstrated by the electron microscope of the pseudorabies virus (Aujeszky strain) from infected rabbit brains.[7]

Table 1 shows the infectivity of the erythrocyte specimen from the test group when injected intracerebrally into Swiss albino mice. Those mice succumbing to the disease developed symptoms of central nervous system involvement within three to five days after inoculation. All 6 mice inoculated from the control group (48 hour) remained normal throughout the observation period. All surviving mice from the control and test groups were discarded after a 21-day observation period.

DISCUSSION

From the results obtained in this experiment, it was apparent that the virus was present in the blood from twenty-four to ninety-six hours postinoculation. Electron microscopy of the virus-like particles showed the particles to be present on the surface of the erythrocyte. These virus-like particles resemble the pseudorabies vi-

Fig. 7—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval of seventy-two hours. Virus-like particles are indicated by arrow. Shadowed with chromium at arc tangent of 3/14. x 30,000.

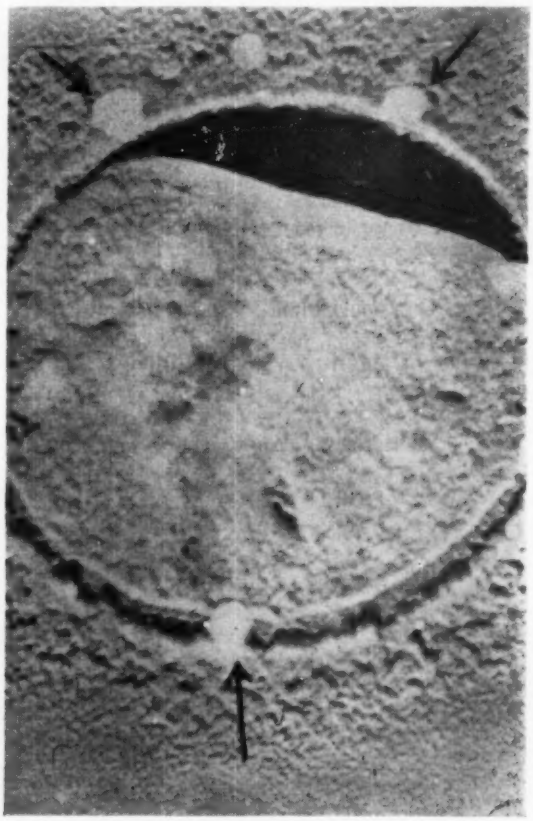

Fig. 8—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval at ninety-six hours. Virus-like particles are indicated by arrows. Shadowed with chromium at arc tangent of 3/14. x 30,000.

rus (Aujeszky strain) in diameter and contour as described in a previous paper.[7]

SUMMARY

Electron microscopic studies were made of erythrocytes from Swiss albino mice injected intraperitoneally with pseudorabies virus (Aujeszky strain). Electron micrographs of erythrocytes obtained from infected mice at 24, 30, 48, 54, 72, and 96 hours postinoculation revealed the presence of virus-like particles resembling in shape and contour those of the pseudorabies virus. The 24-, 30-, 48-, 54-, 72-, and 96-hour erythrocyte specimens were also infectious for Swiss albino mice when injected intracerebrally. No virus-like particles could be demonstrated in the normal controls.

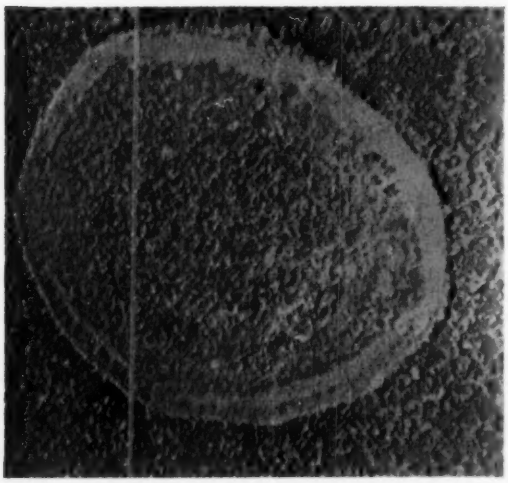

Fig. 9—Electron micrograph of an erythrocyte from mice infected with pseudorabies virus from a time interval of ninety-six hours and showing no virus-like particles. Shadowed with chromium at arc tangent of 3/14. x 20,000.

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Cyanogenetic Compounds in Plants and Their Significance in Animal Industry

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CYANOGEN compounds include substances derivable from cyanogen (C_2N_2), an extremely poisonous, inflammable, colorless gas which reacts in many respects like a halogen. Its hydrogen compound is hydrocyanic acid. In many cyanogen compounds, the univalent group (CN) acts like an element; thus cyanogen is regarded as the radical (CN) which also has the double formula C_2N_2. It has an odor much like the macerated pits or leaves of peach or cherry trees; its compounds have many uses; it is destructive to life, especially animal life. On higher animal life, it affects both the individual cells, such as the important nerve cells, and it also reacts with the hemoglobin of the red blood cells to form cyanhemoglobin. Mazeiter[29] discusses the clinical, anatomical, and experimental features of poisoning by hydrocyanic acid. The first report of hydrocyanic acid in plants was made in 1803.[40]

The following discussion considers how the cyanogen radical combines into a compound as part of a plant or plants which may be harmful to animals, and compiles data from the literature which discusses plants that produce the cyanide radical hereafter referred to as HCN. It is discussed under the following seven headings:

1) Names of plants or groups of plants that are known either to produce HCN sickness and death, or those that are potentially dangerous as stock poisoners; (2) animals chiefly affected; (3) conditions that enhance the production of HCN in plants; (4) the dosage, or the amount required to produce sickness or death; (5) the glucosides or precursors of HCN; (6) symptoms of poisoned animals and remedies for the animals; (7) postmortem findings and histopathology of poisoned animals.

IMPORTANT CYANOGENETIC PLANTS

A great many plants are capable of producing HCN sometime during their growth. Quisumbine[37] lists over 300 separate species distributed into 74 different families of seed-bearing plants which he says are known to contain HCN. The greatest number, 52, are in the pea family (Leguminosae), with 25 in the grass family (Graminae), and 10 or more in each of the aster (Compositae) and spurge (Euphorbiaceae) families. There are references to many other plants in the literature. Under certain conditions, ferns have produced HCN[20] and, when in the young shoot stage, bracken may produce enough HCN to be a possible danger.[32] There are records of HCN from the thallophytes, such as from a fungus,[39] and from mushrooms.[2] No definite reference to HCN in algae was found but O'Donoghue and Wilton,[34] in discussing algae poisoning in Alberta, Canada, report that while the poisonous substance has not been recovered and identified by chemical analysis, HCN is suspected since the intravenous administration of a solution of sodium nitrite and sodium thiosulphate has curative value. Either of these is a most effective remedy for HCN poisoning.

Some of the bacteria which are generally considered belonging to the plant kingdom produce HCN. Clawson and Young[10] gave a preliminary report on HCN production by bacteria. Lorek[25] found that HCN was produced by a strain of *Pseudomonas aeruginosa* (Bacillus). Hurst,[23] observing that well over 1,000 species of plants have been found to be cyanogenetic in at least part of their life history, considers it remarkable that only about 12 cyanogenetic glucosides have so far been isolated.

In the United States, probably the most important plants causing livestock losses from HCN are found in the grass family. The different species of sorghum, including Sudan grass and Johnson grass, are our worst offenders. Even those plants, however, are harmless most of the time and are considered among the very good forage crops for livestock. Bermuda (Cynodon spp.) grass, with a wide geographical distribution, is also potentially dangerous. Other potentially dangerous plants are chokecherry (Prunus spp.) leaves, especially to trailing animals,[27] and arrow grass *(Triglochin maritima)* under drought conditions.[9] According to Steyn,[43] species of Cynodon cause more HCN losses than any other species in South Africa.

There are some wild, native plants that have been shown to contain considerable potential HCN, but only an occasional isolated case of poisoning has been attributed to them. *Suckleya suckleyana*, a weedy chenopodium prevalent in the Rocky Mountain region, especially Colorado,[44] received much attention in the latter part of the 1930's. Seven other new plants in the United States, mostly wild range types that were shown to contain considerable HCN,[33] are *Emplectocladus fasiculatus* (California desert almond), *Linum kingii* (yellow flowered flax), *Linum lewisii* (prairie flax), *Glyceria septentrionalis* (manna grass),

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Centuria Americana (star thistle), *Stillingia dentata* (queenroot), and *Triglochin palustris* (arrow grass).

Minssen [31] makes the interesting suggestion that HCN of manna grass is in the form of an easily decomposable compound other than a glucoside. He states that ensiling manna grass reduces the HCN content. Perhaps *Trifolium repens* (white clover), a common plant in the United States and one that is cosmopolitan, should be mentioned as another HCN plant.[11, 41] Work is now being done in New Zealand to develop strains of white clover that are free from HCN.[13] Of lesser importance from a livestock angle, but sometimes causing poisoning in human beings, are lima beans *(Phaseolus lunatus)* and manioc or cassava *(Manihot utilissima)*.[6] Whether or not all those plants are poisonous to animals is a debatable question. It is true that under certain conditions they might be.

According to Worden [46] in Great Britain, the commonly used linseed cake is probably the most often suspected as a source of poisoning in livestock, although generally on inadequate grounds. Things to be considered when estimating the danger to stock, or to man, besides the potential HCN in these plant foods are palatability, abundance, ratio of enzyme to glucoside in the plant, acclimation of animals to it, and its state of preparation for food, such as raw or cooked.

The following simple test is available for plants that are suspected of containing HCN: Cut narrow strips of filter paper, saturate them with a solution of picric acid, let dry, then just before using dip each piece in a solution of sodium carbonate. If a plant or part of a plant is suspected of containing HCN, a few grams of the plant material, chopped into small pieces, are put into a test tube, a few drops of chloroform are added to facilitate the evolution of HCN, and a strip of the sodium picrate paper is suspended above the plant material in the test tube by fastening one end to the lower part of the cork stopper. Incubate it in any convenient way, such as putting it in the vest pocket for a few hours, and if HCN is present, the yellow sodium picrate paper will turn red. The rapidity and amount of the red color indicates the quantity of the HCN content of the plant material.[22]

Another method described by Brunswik,[4] is to moisten a little of the chopped, suspected plant material, add a little chloroform, place in a covered glass dish, and on the inside of the cover suspend 1 drop each of two different reagents; (1) a 1 per cent silver nitrate solution faintly colored with methylene blue; and (2) a solution of benzidine and copper acetate. If HCN vapor is present, drop (1) will show distinctly bluish crystals of silver cyanide, and in drop (2) blue needle crystals of an oxidation product of benzidine will form.

Möller and Stefansson [30] give methods for determining quantitatively small amounts of cyanide.

If it is necessary to preserve plant material for chemical analysis at a later date, this may be done by saturating and covering the plant material with a 1 to 3 per cent solution of mercuric chloride.[3]

ANIMALS CHIEFLY AFFECTED

Hydrocyanic acid is poisonous to any type of animal because it affects respiration, paralyzes nerves, and interferes with normal physiological functions.[35] Evans and Evans,[17] in their experiments with rabbits, drew attention to the similar effect of HCN and clover juice on isolated rabbit intestine and suggested that one form of bloat may be due to the high HCN content of legumes.

Sometimes HCN is used in therapy because of its anesthetic effect. The direct application of HCN to the unbroken skin produces local anesthesia.[42] It has been used as an anodyne in pruritus and to relieve coughing and vomiting, but is not very effective and the danger of fatal mistakes renders its use inadvisable. It interferes with enzymes which play an important part in life processes and is, therefore, a violent protoplasmic poison for all forms of life whether they be bacteria, infusoria, yeasts, or germinating seeds. In certain fish ova, cyanides suspend the autolytic process accompanying death so that cells remain in a condition between life and death for several days. Although HCN inhibits putrefactive bacteria, it is not a potent bactericide.[42]

Whether there is any chronic effect of HCN on livestock is questionable. When an animal recovers from a sublethal dose of HCN, it is usually considered to have no effect on a later dosage. The HCN plants probably produce no real chronic stock poisoning similar to that of the Senecio spp., locoweeds, or other plants that produce a cumulative effect. Injury to nerve tissue, blood corpuscles, or other tissues or cells which lower resistance of animals to more poison, might be interpreted as a chronic effect.[45] Reed [38] concluded that when human beings are exposed to HCN gas, chronic symptoms occur only on repeated exposure. A severe exposure that is not fatal produces no chronic symptoms. According to experimental results, there are no manifestations of increased susceptibility but, on the contrary, some indication of a certain degree of tolerance. It is

possible that animals may become habituated to HCN. Eckell,[16] in one experiment with a horse, gave daily doses of HCN, starting with small sublethal doses and gradually increasing the dosage, in 20 days, to what was considered a minimum lethal dose without producing symptoms.

One might expect that animals with a simple stomach should be more susceptible to poisoning than ruminants, because acidity which is common in the true stomach is conducive to the breakdown of the glucoside to HCN while alkalinity has the opposite effect.[14] However, acidity destroys the enzyme that produces HCN.[15] Ruminants are more susceptible to poisoning than horses and swine.[15] Sheep are slighly less susceptible than cattle if actual dosage is considered. Some breeds of cattle, such as Herefords, are reported to be less susceptible than others.[24] A possible explanation of why ruminants are more susceptible than animals with a simple stomach is that hydrolysis in the rumen does not necessitate the presence of plant enzymes, since ruminal bacteria can hydrolyze cyanogenetic glucosides very rapidly, and HCN is readily absorbed from the rumen.[12]

CONDITIONS FAVORING CYANOGENESIS

The production of HCN in plants varies with environmental conditions and with the stage of growth as well as with the part of the plant. With sorghum, young plants are more poisonous than older ones; nearly mature sorghum contains no HCN, but this is not necessarily true with arrow grass.[9] With both arrow grass and sorghum, growth under drought conditions is conducive to high HCN. The leaf blade of plants generally contains more HCN than stems, roots, or fruits. Franzke and Hume [18] showed that application of manure and increase of soil moisture, and other factors which favored an increased growth in sorghum strains, resulted in lower HCN content. However, this is not the case with nitrate fertilizers which produce increased growth and increased HCN content.[14] Pickney [36] concluded that in plants grown in greenhouses, the HCN content was in proportion to the nitrate fertilizer he used. Just how HCN or cyanogenetic glucosides are metabolized in the plant is not known. Perhaps Worden [46] is correct in theorizing that cyanogenetic glucosides are a normal intermediary product of plant metabolism and probably a step in the conversion of nitrates, absorbed from the soil, into final plant protein. Thus, in some plants the HCN phase is passed rapidly and such plants are considered free of HCN, while in other plants the HCN phase is prolonged. Perhaps, under adverse conditions, such as wilting or frosting, the HCN phase is prolonged and consequently more of the potential HCN is liberated. Lorck [25] reported that Pseudomonas species producing HCN are the most suitable objects for studying the mechanism of formation of HCN in plants.

QUANTITY REQUIRED FOR POISONING

The minimum lethal dose (m.l.d.) of HCN depends on the rate of absorption into the animal's system. However, with a detoxification occurring continuously, if it should equal the rate of absorption, death would not result regardless of the amount absorbed. According to Clawson, Bunyea, and Couch,[8] the m.l.d. of drenched HCN is about 2.042 mg. per kilogram for cattle and about 2.315 mg. per kilogram for sheep. At this rate, a 40-kg. sheep would be killed by 92.7 mg. of HCN. However, feeding 600 Gm. of plant material containing 18 mg. of HCN per 100 Gm., a total of 108 mg., to a 40-kg. sheep should not be lethal because it would not all be available at one time for absorption. It would be absorbed as liberated and detoxified by the system before a lethal amount could accumulate. Thus, when the ingestion of food is relatively slow, as in normal grazing, the m.l.d. would approach 3.9 mg. per kilogram of animal per hour. This would be 31 mg. per kilogram for an eight-hour grazing period if grazing were uniform throughout the day. However, since this is not the case the actual 24-hour tolerance probably varies between 15 and 50 mg. per kilogram.[12]

The late J. F. Couch was of the opinion that plant material which contained less than 20 mg. of HCN per 100 Gm. was not toxic to animals, but if the plant material contained more than 20 mg. per 100 Gm. of plant material, it was a dangerous feed.

In 1940, stunted arrow grass collected from a dry alkali area in Utah, and analyzed in the laboratory of the U. S. Department of Agriculture, contained as

much as 600 mg. of HCN per 100 Gm. of plant material. Similar material was lethal when 0.4 lb. of the arrow grass was fed to a 100-lb. sheep within a few minutes.[9]

THE GLUCOSIDES, PRECURSORS OF HCN

Free HCN as such is not found in an appreciable quantity in healthy growing plants but develops when normal growth has been retarded or stopped by drought or other adverse conditions. It is usually formed by chemical reaction between two substances, a glucoside and an enzyme, contained in the plant. Neither the glucoside nor the enzyme is poisonous by itself.[14] According to Hurst,[23] the cyanogenetic glucosides are colorless, crystalline substances which, after isolation from the plant, are distinguished by their chemical stability. The glucoside consists of a chemical combination of a sugar and HCN with perhaps another compound, probably an aldehyde or ketone. Only when liberated is the HCN poisonous. The remedies, mentioned later, usually combine with HCN to make harmless compounds.

Cyanogenetic glucosides studied include amygdalin, lotusin, dhurrin, sambunigrin, linamarin, and lotaustralin. Of these, the best known and most extensively studied is amygdalin, which was isolated in 1830 from the bitter almond and related plants. Lotusin is isolated from the Nile Valley plant called "khuther" *(Lotus arabicus)*. Dhurrin, first isolated from sorghum in 1902, was one of the first cyanogenetic glucosides studied, according to Henry.[21] Sambunigrin is derived from elderberries. Linamarin, formerly called phaseolunatin, was first isolated from the seeds of uncultivated lima beans in 1903.[6, 21] It also occurs in flax. Lotaustralin occurs in white clover *(Trifolium repens)*.[12] Much recent work has been done on arrow grass but no glucoside has been isolated.

SYMPTOMS AND TREATMENT OF POISONING

Livestock poisoned by HCN show signs of suffocation which may be manifested in several different ways. Usually the first signs are increased respiration and pulse rate. These may be followed by hypersensitivity to stimuli, spasmodic muscular movements, respiratory convulsions, and dyspnea.[5] Several remedial or prophylactic measures are reported but, because of the rapid action of HCN, are usually not practical. The treatment which has proved most effective in saving sheep, poisoned experimentally with either HCN or plants containing HCN, is a solution of sodium nitrite and sodium thiosulfate given intravenously or intraperitoneally.[5] Treatment with methylene blue and sodium tetrathionate are also discussed by Bunyea.[5] Other therapeutic measures mentioned are amyl nitrite for human cases;[7] oxygen and artificial respiration for rabbits;[19] cobalt nitrate[26] and copper chloride injection.[1] Then as a preventive, glucose may be given by mouth.[14] There is a quantitative limit for drugs, such as nitrites, that produce methemoglobin which readily forms cyanmethemoglobin and uses up the cyanide. The quantitative limit would be approximately the quantity required to combine with three to four fatal doses because, although the cyanmethemoglobin formed is not toxic in itself, it, like methemoglobin, is not an oxygen carrier. The blood can not function in respiration if a too great amount of its hemoglobin is in the form of cyanhemoglobin, cyanmethemoglobin, or methemoglobin.

PATHOLOGY AND HISTOPATHOLOGY

At postmortem examination, there is no characteristic lesion which would definitely point to HCN poisoning. Occasionally, when sheep are poisoned by cherry leaves, there is a characteristic benzaldehyde odor if the rumen is opened soon after death. The most common characteristics are dark muscle tissue, congestion or hemorrhage of the lungs, petechiation of the tracheal mucosa, and a frothy, bloody discharge from the mouth and nostrils.[5] Microscopic study of the tissues of animals poisoned by arrow grass showed very little injury to the tissues or cells. There was slight evidence of irritation in the kidneys, possible injury to the hepatic cells, and some evidence of mild capillary congestion in the lungs and walls of the heart. Unusual quantities of hematogenous pigment in various places was suggestive of the degeneration and destruction of many red blood corpuscles.[28]

SUMMARY

This paper discusses plants that may be harmful because of their cyanogen content, the animals that are most likely to be

harmed, the condition and amount necessary to produce sickness, the precursors of the hydrocyanic acid in plants, the symptoms produced, possible remedies, and postmortem findings of poisoned animals.

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Addendum

NOTE ON REMEDIES FOR HCN IN PLANTS

In theory, hydroxycobalamine (vitamin B_{12a} or vitamin B_{12b}) may be another remedy for livestock poisoned by hydrocyanide acid (HCN) because it reacts readily with HCN to form cyanocobalamine (vitamin B_{12}) and water. This reaction is irreversible and the cyanide seems to be tightly bound within the coördination complex of vitamin B_{12} which is indicated by the observation that dose levels of vitamin B_{12} up to 1,600 mg. per kilogram both intraperitoneally and intravenously are nontoxic to mice. The 1,600 mg. of vitamin B_{12} contains about 32 mg. of HCN or at least eight times the lethal dose for mice.[1] There is about 2 per cent in vitamin B_{12} according to Wijmenga and Hurenkamp.[3] Cyanocobalamine (vitamin B_{12}) contains one cyano group linked to the cobalt atom whereas hydroxycobalamine (vitamin B_{12a} or vitamin B_{12b}) carries a hydroxyl group instead of a cyano group.[2]

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